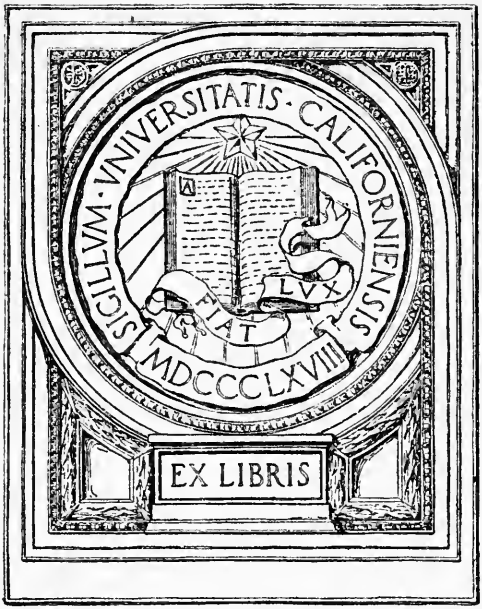
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Laboratory Technique

The Methods Employed at St. Luke's Hospital

NEW YORK

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and
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409 West 59th Street,
New York City

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THE METHODS EMPLOYED AT ST. LUKE'S HOSPITAL

F. C. Wood, Karl M. Vogel,
and
L. W. Famulener.

With the extraordinary development of technical methods, the hospital laboratory to-day is called upon to perform work of an extremely complicated nature in much greater amount than was the case ten years ago. In fact, the total volume of work is so large that unless some definite system is employed mistakes are certain to occur. Moreover, it is important that the laboratory procedures be standard ones, that the limits of accuracy be thoroughly understood, and that the methods of carrying out the tests do not vary from year to year with the shifting of the laboratory internes or of the department chiefs; in this way only can the reports be of value to the attending physicians. For example, since there is considerable variation in the readings obtained with the Tallqvist, Sahli, and Fleischl-Miescher hemoglobinometers, it is necessary for diagnostic and therapeutic purposes that the clinician know with what apparatus a hemoglobin determination has been made. In a case of doubtful syphilis, also, it is very important that the physician understand whether the Wassermann test has been done according to the original method, by which a certain small percentage of late syphilides give no reaction, or with one of the highly sensitive cholesterinized antigens, by which a faint reaction may possibly be obtained in a person not afflicted with the disease.

In attempting, therefore, to standardize the laboratory work in the Pathological Department of St. Luke's Hospital, there was prepared some time ago a series of small typewritten notebooks, containing the necessary directions for the examination of blood, urine, sputum, etc., methods of preparation of pathological material, and the simpler bacteriological and serological procedures. In the course of time these notebooks have been

expanded; and, as interest in the technical methods used at St. Luke's has been expressed by workers in other hospitals, it has been felt that perhaps a wider circle can be reached by the publication of our laboratory procedures in the Hospital Report. While the directions given are of value chiefly to the interne and to the recently graduated physicians, who are apt to be the persons doing most of the laboratory work in connection with patients, it is hoped that they may also prove a useful guide for older practitioners whose active participation in laboratory work is only occasional.

Inasmuch as these are merely laboratory notes, no attempt has been made to furnish references to the sources from which the procedures have been obtained, and full acknowledgment is hereby made to the numerous authors on whose contributions the following descriptions are founded. Many of the tests as given are modifications developed in the laboratory from the original procedures, and are here published for the first time. But it is not presumed that these are the only methods which might or should be employed. The methods given here represent merely the practice at St. Luke's laboratory, and are such as we have found satisfactory after long use. The somewhat peremptory and dogmatic style in which the directions are written is explained by the fact that they have been prepared for the use of internes in the pathological laboratory, as stated above, and therefore, have been compressed as much as possible, all irrelevant matter being omitted.

The writer is responsible for the chapter on histological methods, and, in conjunction with his colleague, Dr. Karl M. Vogel, has revised the notes on clinical pathology. Dr. Vogel bears the entire responsibility for the chapter on clinical analytical methods; and Dr. L. W. Famulener has prepared the sections on bacteriology and serology.

F. C. WOOD.

HISTOLOGY

PREPARATION OF HISTOLOGICAL SPECIMENS

GENERAL DIRECTIONS.

Specimens from the operating room are put into jars of 4 per cent. formalin, or, if large, are wrapped in moist towels or gauze, and placed in the ice-box. The date, the patient's name, (surname first), the ward, the name of the operator, and the nature of the specimen are entered in the day-book. (The assistant attending surgeon's name is used only when cases are sent special to him. The house surgeon's name is never used). The name, the ward, the date, and the name of the operator are entered on description card, name card, and diagnosis card (Fig. 1). The day-book number is placed on the jar, and on the three cards. A brief macroscopical description is written on description card (Fig. 1, A), and relations, dimensions of tumors, etc., are given.

One or more pieces for section are cut from the specimen and placed in small bottles of 5 per cent. formalin with the number of specimen on the label. This should be done immediately on the receipt of specimens too large to preserve whole; such large specimens should be submitted to the pathologist to determine whether they are to be preserved in Kaiserling or not. The pieces removed should include the characteristic portions, and if the lesion involves the skin or mucous membrane, the plane of incision should be at right angles to the surface. In all tumors, care should be taken to obtain portions of the adjacent lymph nodes if they are present. When the uterus and adnexa are sent down, there should be cut at least one block from the uterus showing endometrium, one from each tube, and one from each ovary. Unless otherwise requested by the surgeon, all large specimens should be divided like a book with parallel cuts about one inch apart, running nearly through the specimen for distinctive portions, and for proper penetration by the fixative. The

Fig. 1. Cards used in recording surgical material (about half size). A, Description card, filed according to serial number. B, Diagnosis card, filed according to anatomical region. C, Name card (blue) filed alphabetically.

blocks are best cut roughly for formalin, and, after they have been in the 95 per cent. alcohol, trimmed to pieces 3 to 5 mm. thick, and not much over 2 cm. in diameter.

Tissue blocks are then embedded as follows, unless they contain bone or calcified areas. For large tissue blocks for which there is no hurry in making a report:

1. 4 per cent. formaldehyde, 12 to 24 hours;
2. 80 per cent. alcohol, 2 hours;
3. 95 per cent. alcohol, 2 hours;
4. 95 per cent. alcohol, overnight;
5. Absolute alcohol, two changes in 24 hours. The number of changes will depend upon the bulk of the blocks in the bottle. If only one or two small blocks are to be dehydrated, two changes of a small quantity of alcohol will suffice. If the bottle is half full of tissue, it will be best to make three changes;
6. Equal parts of carbon disulphide and absolute alcohol. The blocks should sink in this mixture and become transparent. When this occurs, they are sufficiently penetrated. For a thin specimen only 1 hour may be required; for thicker specimens 3 to 4 hours. If the blocks do not clear up, they still contain water and must be returned to the absolute alcohol for further dehydration;
7. Pure carbon disulphide, 2 to 6 hours. The blocks float in this at first and become somewhat cloudy. Usually they sink to the bottom after some hours, but occasionally not at all. If the specimen contains a large amount of fat, two changes of carbon disulphide should be used. The alcohol being removed by the carbon disulphide, the preparation is transferred to
8. Carbon disulphide saturated with paraffin at room temperature. Such a solution contains 16 to 20 per cent. of paraffin. In this the blocks sink and again become quite transparent, and the material can remain in such a solution for months without deterioration. This step can be omitted if haste is necessary, and the blocks transferred at once to
9. Carbon disulphide saturated with paraffin at incubator temperature, 37 to 40 C. This contains 68 gm. of paraffin to 100 c.c. Moderate sized blocks should be left in this 37 C. carbon disulphide and paraffin for 1 to 2 hours if the tissue is very thin,

or for several days if more than 1 cm. thick. Prolonged treatment at this temperature does not make the tissues hard or brittle. When the blocks are perfectly clear, which usually occurs in four to six hours in routine specimens, they are transferred to

10. Stock melted and *filtered* paraffin at 55 C. for 1 hour, then to a fresh lot of paraffin, not previously used, for 1 hour.

11. A shallow tin dish is filled with the freshly melted paraffin not previously used for embedding purposes, a strip of paper is laid on the bottom, carrying the number of the specimen in pencil, and the pieces of tissue are arranged in the dish with a hot needle. It is convenient to place on the bottom of the dish the surface from which the section is to be cut. Bubbles can be removed with hot needle. If a skin forms over the surface, it can be removed by a moment's flaming with a Bunsen flame. When the blocks are all arranged satisfactorily in the dish, it is floated immediately on cold water so as to cool the paraffin as rapidly as possible. If the inside of the dish has previously been rubbed with a trace of glycerin, the paraffin will separate from the walls easily. If not, the blocks must be cut out with a knife. The dishes are conveniently kept in the ice-box, as the contraction may cause the paraffin to crack away from the walls and so free the block without cutting.

Care must be taken that the specimen is never left in the paraffin at 55 C. for more than two hours, as the tissues are very much injured by heat. If the specimen contains any water, the heat of the paraffin bath will ruin it.

In cutting out such embedded blocks, a thin-bladed knife should be used, and the material should be sawed rather than cut, because if direct pressure is exerted the block may crack in a number of directions, and the cracks pass through the tissue. Each piece of tissue is cut from the cake and trimmed carefully, always leaving a wall of paraffin, the walls of the block being cut perpendicularly to the surface and at right angles to each other. The trimmed tissue and paraffin are fastened to a fiber block after the surface of the latter has been heated in a Bunsen burner or over an electric stove, the thickest side being placed against the hot block, where it will adhere owing to the melting of the paraffin. Care should be taken that the tissue itself is not heated by the hot fiber, as this will interfere with the staining.

The fiber block is immediately numbered with a lead pencil. After it has cooled, the surplus paraffin is trimmed close to the sides of the tissue, leaving a little more at the ends to permit of satisfactory ribboning of the specimen. The blocks can be kept in the ice-box until they are to be cut, when they are clamped in the microtome with the long diameter of the block perpendicular to the edge of the knife.

The microtome knife should be stropped and placed at an angle of about 15 C. to surface of block, and adjusted so that it just clears the block. The feed is adjusted to give four clicks (about 13 micra in the International Instrument Company's Minot model microtome), and thick sections are cut until the whole piece of tissue appears in sections. The feed is then adjusted to two clicks (about 7 micra); a new portion of the knife is selected; and ribbons of sections are spread on a pan of distilled water at about 48 C. on electric stove. Even, flat sections are fished to two or three clean slides, and the slides are numbered with a diamond pencil and stood on end to drain. If desired, in order to prevent the sections from coming off the slides, the slides may be rubbed over with a very small amount of egg albumin solution with the finger before mounting the sections. They are then placed in trays in the oven at 37 C. for twelve hours to dry (unless "rush"; see special technique). The slides should be covered by a sheet of paper or cardboard to prevent dust settling on the preparations. When dry the paraffin should be melted by placing the tray in the oven at 55 C. for a few minutes. After cooling the slides are transferred to a jar of xylol.

Sections are prepared and stained as follows:

1. Xylol, about 2 minutes;
2. Xylol, about 2 minutes;
3. Alcohol absolute, 2 minutes;
4. Alcohol absolute, 2 minutes;
5. Alcohol, 80 per cent., 2 minutes;
6. Water, 2 minutes;
7. Hematoxylin, about 5 minutes (control with microscope);
8. Distilled water, 10 minutes;
9. Tap water, 10 minutes (better 1 hour);
10. Alcohol, 80 per cent., 2 minutes;

11. Eosin, ½ to 2 minutes;
12. Alcohol absolute, 2 to 3 minutes;
13. Alcohol absolute, 2 to 3 minutes;
14. Alcohol absolute, 2 to 3 minutes;
15. Carbol-xylol, 1 minute;
16. Xylol, 1 minute;
17. Mount in gum dammar; label with number.

The diagnosis is written on the blue name card (Fig. 1, C) by the pathologist, and signed; it is then copied exactly by the interne on the description card (Fig. 1, A) and the white diagnosis card (Fig. 1, B), and in the day-book. A duplicate of the diagnosis card is filled out and sent to the attending surgeon.

A brief microscopical description is written by the interne on the description card, and handed to the stenographer to be copied. When returned, the card is filed by number. The small white card is filed according to anatomical region, the blue card by name.

If a previous specimen from the same patient has been examined, the number of the former entry should be noted on the blue card and in the day-book before the section is presented for diagnosis. One slide of each representative block (or more if of special interest), is filed in the slide cabinet by number.

Gross specimens are invariably kept until after the diagnosis is signed. Specimens too large for preserve jars are tied in gauze with a permanent tag bearing their numbers in lead pencil, and placed in crocks or anatomical jars. A special crock is kept for fibroids, and another for breast tumors. When the attending pathologist signs the diagnosis, he should be asked what disposition is to be made of the specimen. If it is thrown out, the day-book entry should be stamped "Spec. not saved." The blocks also are kept until the diagnosis is signed. If saved, they are labeled with a paster bearing number, and filed in numbered pasteboard boxes.

The interne histologist is responsible for:

Care of surgical specimens, and cutting and staining of sections.

All records of such specimens and the report to the operator.

Care of microtome, paraffin, knives, and stains.

No diagnosis is to be reported unless signed by the attending or resident pathologist.

ZENKER'S FIXATIVE.

When Zenker's solution is used for fixation, the changes in the technique are as follows: Specimens not to be over 5 mm. in thickness.

Place in Zenker's solution for 6 hours.

Wash in running water for 24 hours in a Fairchild porcelain cup or a wide-mouthed bottle covered with gauze, through which a glass tube drawn to a point is thrust. The tube is connected with the water supply and a slow stream is allowed to flow in. Proceed as after formalin above.

In staining: After the 95 per cent. alcohol wash for 10 to 20 minutes in tincture of iodine to remove the mercuric chloride, and then wash, till all brown color is removed, in 95 and 80 per cent. alcohol. A treatment with a 5 per cent. aqueous solution of sodium hyposulphite (fixing hypo) will remove the iodine very completely. Wash in distilled water, then follow regular routine. It is often difficult to stain Zenker's preparations with hematoxylin; if trouble is experienced, place the section over night in a very dilute (1:50) Delafield's hematoxylin and decolorize with 1 per cent. hydrochloric acid alcohol if necessary. Or, if preferred, Weigert's iron hematoxylin can be used for the usual length of time.

CARE OF KNIVES.

Honing.

Microtome knives are honed on a yellow Belgian water hone, used for them only. Wet the surface with water or water and glycerin in equal parts. Hone against the edge from heel to toe, always making the full stroke. The knives are hollow ground, so keep the back in contact with the stone and do not press the blade against the stone, merely steady it with the fingers of the left hand. Hone until the edge, or a usable portion of it, is free from nicks when examined under the microscope with a low power. If the knife has been injured by contact with bone, hone on a so-called carborundum razor hone, using either soap and water or glycerine and water until the nicks are removed, then finish on the Belgian hone. Hone the knife about once a week.

Stropping.

Strop away from edge from toe to heel, also keeping back

edge in contact with strop. Strop until edge appears as straight line with polished borders, and no serrations. Strop between every ten blocks.

Wipe knife with xylol and put in case after using. A small amount of diamantine powder No. 2 may be rubbed into the strop occasionally to increase the sharpening power. When the knife is in proper condition it should easily cut a loosely held hair.

SOLUTIONS.

The staining and clearing solutions are kept in a series of Hellendahl or Coplin staining pots.

Ninety-five per cent. alcohol: kept in three Hellendahl jars. Jar No. 1 is emptied daily into waste bottle, for redistillation of alcohol. No. 2 is emptied into No. 1, and No. 3 into No. 2; and No. 3 is then filled with fresh alcohol.

Absolute alcohol: kept in original bottle or in Coplin jars. Staining jars before carbol-xylol are refilled every week; after second xylol, every two weeks.

Eighty per cent. alcohol: made by diluting 95 per cent., 84 c.c. to every 100 c.c.

Carbon bisulphide *technical.*

Carbon bisulphide and absolute alcohol: made by mixing equal parts.

Carbon bisulphide and paraffin: made by saturating carbon bisulphide with paraffin at 37 C.

Carbon bisulphide is very inflammable. Do not put it in the oven, or carry a lighted match near the bottle. All bottles containing this substance are to be kept *loosely* corked so that any vapor which may be generated from the heat of the room will not blow out the cork.

Carbol-xylol: Stock made by mixing carbolic acid crystals one part and xylol three parts by volume. In order to measure the carbolic acid, it may be melted by standing the bottle in hot water. It should be removed in three days to one week.

Dammar: made by dissolving gum dammar in xylol and neutralizing by shaking up the solution with precipitated chalk. The xylol is frequently acid in reaction.

Eosin stock solution: made by saturating 95 per cent. alcohol with precipitated eosin. The latter is obtained by precipitating

Grübler's "Eosin wasserlöslich gelblich" from a strong aqueous solution with hydrochloric acid; and then filtering and washing the precipitate until it is acid free. The contents of the filter are allowed to dry at room temperature, powdered, and preserved in a stoppered bottle. For use, the stock solution is diluted with 95 per cent. alcohol about 1:10. The stain will keep indefinitely and the jar may be filled up as the solution evaporates.

Hematoxylin: made by diluting stock Delafield hematoxylin 1:10 with water. The solution keeps for a week or two, but must be filtered daily.

Ammonia water: one drop of strong ammonia in jar of distilled water.

Thin celloidin: a 4 per cent. stock solution diluted 1:10 with equal parts of 95 per cent. alcohol and ether.

SPECIAL TECHNIQUE.

FROZEN SECTIONS.

When a frozen section is ordered for an operation, notify the attending or resident pathologist at once. Have ready a tank with plenty of carbon dioxide, a sharp chisel knife, a tray of water, needles, smooth pointed forceps, a camel's hair brush, four clean slides and covers, two glass "tooth powder dishes", a bottle of saturated aqueous solution of thionin, and smooth surfaced blotting paper.

Place the specimen on a freezing microtome attached to the carbon dioxide cylinder; allow the gas to flow for a few seconds; then turn it off for half a minute, and repeat this process, twice if necessary. Take care to avoid freezing the specimen too hard, as the knife cannot cut through the tissues when they are overfrozen. If over-freezing does occur, allow the upper surface of the specimen to thaw until the proper consistency for cutting is reached. Hold the knife at an angle of 30 to 40 degrees above the horizontal, and cut a number of sections rapidly, shaking them from the blade into water. With fine-pointed forceps, seize a suitable section and dip it, for two seconds, into a dish containing 0.5 per cent. aqueous thionin solution; then still holding it with the forceps, remove the section to a dish of water and wash it free of excess of dye. Then slip a slide under the section in the water and gradually raise it, so as to leave the tissue floating

in a little pool on the slide. When the section is perfectly flat, remove the water by applying the edge of a strip of blotting paper to the border of the drop, not touching the tissue itself. Place a cover-glass over the preparation, and examine it with a microscope. The nuclei are stained blue, the connective tissue pinkish, the mucous tissue pink, and colloid blue.

The section can be preserved for a few days by mounting it in Bruns' glucose medium, which is made by dissolving 40 gm. glucose and 10 c.c. glycerin in 140 c.c. distilled water.

"Rush" specimens for permanent mounts may also be cut frozen as follows: After hardening overnight in formalin, cut block not over 1.5 cm. square, wash, place on freezing microtome table, freeze, and cut. Fish thin sections on to slide under water with needle, drain, flood with absolute alcohol, blot hard, and cover with thin celloidin. Then soak the sections in water, stain with hematoxylin, and dehydrate as usual, but use only 95 per cent. alcohol, not absolute alcohol.

When it is desired to block rush specimens in paraffin, the regular routine may be shortened as follows. Very thin small blocks must be used.

		Routine	Rush
1	4 per cent. formaldehyde	12 hrs.	15 min.
2	80 per cent. alcohol	2 "	
3	95 per. cent. alcohol	2 "	15 "
4	95 per cent. alcohol	12 "	
5	1st absolute alcohol	12 "	15 "
6	2nd absolute alcohol	12 "	30 "
7	Carbon bisulphide and alcohol (until block sinks)	4 "	10 "
8	Carbon bisulphide (or until clear)	6 "	30 "
9	Carbon bisulphide and paraffin (cold)	4 "	
10	Carbon bisulphide and paraffin (hot)	12 "	10 "
11	1st paraffin	1 "	30 "
12	2nd paraffin	1 "	30 "
13	3rd paraffin	embed	embed

BONE SECTIONS.

Tissue containing bone or calcareous masses, after thorough fixation in formaldehyde or alcohol, is washed for a short time in water, then placed in a solution of strong nitric acid (7.5 c.c. of acid in 92.5 c.c. of water); which is changed every few hours until no bone can be detected on piercing the tissue with a fine needle. The tissue is then washed for 24 hours in running water, using a 2-way cork in bottle, and embedded in the ordinary way, beginning again with 80 per cent. alcohol. If the staining qualities of the tissue are poor, it may be soaked in dilute ammonia water for ten minutes. Sections are cut with a microtome knife with a heavy blade, especially kept for this purpose so as to avoid injuring the one used for regular work.

STAINS.

DELAFIELD'S HEMATOXYLIN.

The solution is made as follows:

100 c.c. of saturated solution of ammonium alum.*

1 gm. of hematoxylin dissolved in 6 c.c. of 95 per cent. alcohol.

The mixture forms a purple solution which must be exposed to light and air for about a week, until it becomes somewhat more reddish. Then 25 c.c. each of glycerin and commercial methyl alcohol are added as preservatives. The solution is filtered and stored in stoppered bottles. The stain keeps for about two years in well filled, tightly stoppered bottles, ripening slowly and becoming more powerful and also more acid from decomposition of the alum. If the solution must be ripened quickly it may be exposed in a layer 2 cm. thick in a dish covered with filtered paper for three days. When diluted 1:10, this solution will stain nuclei is about fifteen minutes, whereas the same solution kept in a loosely stoppered bottle at the end of three months will stain nuclei the same depth of color in from two to three minutes.

Delafield's hematoxylin should never be used in concentrated form, but should be filtered and diluted from five to twenty times with *distilled* water. The solution so diluted does not keep for

*About 11 per cent. of the crystallized salt.

more than ten days, and an iridescent scum is apt to form on the surface. This scum should be removed by filtration as soon as it forms, for it may adhere to the surface of the sections as a blue or black deposit, and ruin them.

Sections must always be rinsed in *distilled* water after staining, as tap water usually causes a precipitate of black particles on the surface of the sections. After such rinsing, the sections are improved by a thorough washing in tap water, which by virtue of its slight alkalinity removes any acid from the tissues, and alters the color of the nuclei from a violet to a deep blue.

A solution which requires no ripening may be obtained by using hematin (Grübler) in place of hematoxylin, though even this solution does not reach its full strength for some days.

The hematoxylin may also be oxidized into hematin, as suggested by Harris, by adding 0.5 gm. of mercuric oxide to each 100 c.c. of the freshly made alum-hematoxylin solution and boiling. As soon as the liquid becomes a dark reddish purple color it is removed from the flame and cooled rapidly. The glycerin and methyl alcohol are then added. The stain may be filtered and used immediately.

If a pure nuclear stain is desired, the addition of 2 c.c. of glacial acetic acid to 100 c.c. of the Delafield hematoxylin will give an acid hematoxylin which practically does not stain the cell body. Or, if preferred, the sections may be left in Delafield's hematoxylin until they are slightly overstained, and then the excess of dye removed by decolorizing with acid alcohol containing 1 per cent. of hydrochloric acid in 95 per cent. alcohol.

WEIGERT'S STAIN FOR ELASTIC TISSUE.

1 Lithium carmin, 10 to 20 minutes;
2 Acid alcohol, 2 to 10 minutes;
3 Weigert's elastic tissue stain, 25 to 30 minutes;
4 Alcohol;
5 Absolute alcohol, 2 to 18 hours, to differentiate;
6 Xylol; balsam (do not use carbol-xylol).

Preparation of Stain

Basic fuchsin	2 gm.
Resorcin	4 gm.
Water	200 c.c.

Heat mixture in porcelain dish; and when boiling add 25 c.c. of liquor ferri sesquichloridi. Filter the solution and allow the precipitate to drain until nearly dry. Return filter paper to same dish, and cover with 200 c.c. of 95 per cent. alcohol; boil; filter; make up to 200 c.c. with 95 per cent. alcohol; and add 4 c.c. of concentrated hydrochloric acid.

WEIGERT'S IRON HEMATOXYLIN STAIN.

After sections have gone through the xylol, alcohol and distilled water, mix equal parts of Weigert's No. 1 and No. 2, and stain for 5 to 10 minutes. Wash thoroughly in several changes of water for half an hour, then put through eosin, the alcohols, and xylols, and mount in gum dammar. In the original method a picro-acid fuchsin counterstain was used, but this fades rapidly. This is the best stain for old material which has been kept for a long time in alcohol. Sometimes decalcified bone will stain by this method.

FAT STAINS.

The most satisfactory method to demonstrate fat is that employing some of the anilin dyes. Of these Sudan III and Scharlach R are the most suitable. The tissues are fixed in 4 per cent. formaldehyde or formalin (Müller's solution), and, after cutting on a freezing microtome, the sections are transferred to 50 per cent. alcohol, and then to the staining solution. This consists of a saturated solution of either of the above mentioned dyes in a mixture of equal volumes of acetone and 70 per cent. alcohol. Usually ten or fifteen minutes are sufficient for staining. Sections are then rinsed in alcohol of 60 to 70 per cent. strength and transferred to distilled water. If desired, nuclei can be lightly tinged with dilute Delafield's hematoxylin. The sections may be mounted in glycerin jelly or levulose syrup.

Fat particles are colored orange by Sudan III and red by Scharlach R. Fatty acid crystals do not stain. Myelin, lipochrome, lecithin, and "protagon" droplets are also stained.

MARESCH-BIELCHOWSKY STAIN FOR CONNECTIVE TISSUE FIBERS.

Tissues are fixed in formaldehyde and embedded in paraffin. After cutting the sections are treated as follows:

1 Placed in 2 per cent. silver nitrate solution, 12 to 24 hours;

2 Washed quickly in distilled water, 5 seconds;
3 Stained in ammoniacal silver solution, 2 to 30 minutes; according to thickness of section;
The solution must be prepared fresh, as follows: To 20 c.c. of 2 per cent. silver nitrate solution add 3 drops of 40 per cent. sodium hydrate solution, and then add ammonia drop by drop until the precipitate is dissolved.
4 Washed in distilled water;
5 Reduced in 8 per cent. formaldehyde until section is colored gray or gray brown; (Reduction takes place place almost instantly.)
6 Placed in gold bath, 10 minutes;

1 per cent. aqueous solution of gold chloride,	3 drops
Distilled water,	10 c.c.
Glacial acetic acid	3 drops

7 Placed in 5 per cent. solution of hyposulphite of soda, 15 to 30 seconds;
8 Washed in distilled water;
9 Fixed in slide with albumin;
10 Dehydrated in alcohol;
11 Cleared in xylol;
12 Mounted in dammar.

STAIN FOR TUBERCLE BACILLI.

1 Delafield's hematoxylin, 20 to 30 minutes;
2 Water, 30 minutes;
3 Carbol fuchsin, 1 hour at 37 C. (incubator);
(Place slide in Petri dish and flood it; then cover to prevent evaporation.)
4 Decolorize in acid alcohol, 1 minute;

Hydrochloric acid	1 c.c.
95% alcohol	70 c.c.
Water	30 c.c.

5 Wash in 70 per cent. alcohol, 2 to 3 minutes;
6 Wash in water;
7 Lithium carbonate solution until section takes blue color;

Sat. sol. lithium carbonate	1 part
Water	10 parts

8 Wash in water, 5 minutes;
9 95 per cent. alcohol, 5 minutes;
10 Absolute alcohol, 5 minutes;
11 Xylol, 5 minutes;
12 Mount in gum dammar.

To obtain the best results the material should be fixed in 95 per cent. alcohol, though formalin preparations occasionally stain well. After fixation in Zenker's solution, the bacteria do not stain satisfactorily.

CLINICAL PATHOLOGY

BLOOD.

A "leucocyte count" includes an estimation of the number of leucocytes, based on a count of 400 small squares at a 1:20 dilution, multiplying factor 200. If the number of leucocytes is above 50,000, a 1:100 dilution may be used or only 100 squares counted. A differential count of 200 cells is made on a slide stained with Jenner. If the count is 9,000 or below, 100 cells are sufficient for a differential except in cases of special interest. In making red or white cell counts or differential counts the use of an automatic tally register (obtainable under this name in sporting goods stores) is of service. In making a differential count, record on the instrument each leucocyte seen, and note, either mentally or on paper, all varieties except polynuclears. When the total reaches 100 or 200, as the case may be, the number of polynuclears is obtained by subtracting the sum of all the other varieties from the total number.

In routine differentials the cells are classified as follows:

Polymorphonuclears: Cells with irregular nuclei and neutrophile granules and forms with kidney-shaped nuclei and neutrophile granules.

Lymphocytes: All mononuclear cells not distinctly granular.

Eosinophiles: Polymorphonuclear cells with eosinophilic granules.

Basophiles: Polymorphonuclear cells with basophilic granules.

Large mononuclears: Cells with large, pale, oval nuclei and no granules with the Jenner stain. If abundant they should be separately enumerated. If not, they may be included with the lymphocytes.* Transitionals resemble the foregoing but have

* Strictly speaking, the large mononuclears and transitionals belong with the myeloid group of cells, since they give the oxidase reaction.

kidney-shaped or somewhat irregular nuclei. In counting they are grouped with the large mononuclears.

Myelocytes: Cells with large, pale, round or oval nuclei and neutrophilic, eosinophilic, or basophilic granular protoplasm.

The usual form of record is:

"W. B. C.: 12,500, Polys: 78, Lymph.: 21.
Eos.:1/2, Bas.: 1/2"

In cases in which the blood picture is of special interest, as in leukemias, severe anemias, malaria, etc., the large mononuclears and transitionals should be noted and charted in separate groups.

A "red count" includes an estimation of the number of cells per cubic mm. based on a count of at least 100 small squares at a 1:200 dilution for approximately normal blood or 1:100 for anemic blood, and a description of the morphology of the cells based on examination of a smear stained with Jenner. Note inequality in size (anisocytosis), irregularity in shape (poikilocytosis), or any increase in central depression. Look for abnormally large cells (macrocytes), or abnormally small cells, (microcytes), for cells that stain diffusely blue, i. e., polychromatophilia, or show basophilic granulations, or larger basophilic particles (Howell-Jolly bodies).

In anemias, look for nucleated forms which are classified as normoblasts (cells 10 micra in diameter or less), and megaloblasts (cells over 10 micra in diameter). Large and atypical forms are often found along the borders of the smear and at the end where the last cells are deposited.

A "hemoglobin determination" consists in an estimation made with the Oliver or Fleischl-Miescher instrument.

A "malaria hunt" consists in a careful search of thin smears stained by Wood's stain, using an oil immersion lens and a mechanical stage, and may be considered as negative if no parasites are found after fifteen minutes. If quinine has been given, a longer search may be necessary or thick decolorized smears may be studied. (See Wood: Chemical and Microscopical Diagnosis, 3rd ed., 1912, p. 87).

TECHNIQUE.

Leucocyte count: (See Wood: Chemical and Microscopical Diagnosis, p. 45). Blood is obtained from finger tip or ear by punctur-

ing with a Hagedorn needle. When not in use the needle is kept with point immersed in a bottle of 95 per cent. alcohol. The skin of the patient is cleansed with alcohol before making the puncture. The puncture must be large enough so that slight pressure at a distance of one-half inch causes a large drop to appear. The first drop is wiped away, and the second used.

Blood is drawn in a 1:10 pipette, exactly to the 0.5 mark. The pipette is held horizontally and at right angles to the line of vision. For accurate work the blood cannot be drawn beyond the mark and then blown out. Still holding the pipette in a nearly horizontal position, the tip is wiped with the finger and dipped into a bottle of 2 per cent. acetic acid held tilted on its side. The acetic acid must be filtered every few days. The acid is drawn up until the mixture reaches the 11 mark, rotating the pipette slightly. As soon as the bulb begins to fill, the pipette is changed to a vertical position to avoid including a bubble. When filled, the pipette is gently shaken for half a minute, with a finger held firmly over the end. If a second drop is taken from the pipette it must be again shaken for half a minute. Two or three drops are then allowed to flow out so as to empty the stem, and a small drop is placed in the center of the counting table. Time may be saved by making two chamber preparations, then if one is not satisfactory, the other may be. The drop should be just large enough to cover the table when flattened with a cover slip. It must on no account be large enough to spread beyond the gutter and wet the rim on which the cover rests, or so small as not to cover the rulings. If the cover glass and counting chamber are clean and in optical contact, Newton's rings will be visible when the chamber is held at the level of the eyes. In handling the filled chamber be careful always to keep it horizontal. The presence of an air bubble in the preparation makes it unsatisfactory for counting. A moment is allowed for the cells to settle and the number on one square millimeter (one of the nine large squares into which the Zappert-Ewing field is divided) is counted, using a number 3 objective and a number 3 eye-piece. Cells touching the left or upper boundary line are considered in the square, those touching the right or lower boundary as out of the square. Count at least four square millimeters, preferably in two different preparations, divide the total number of cells by the number of square mm. counted and multiply by 200 to obtain the number

per cubic mm. of undiluted blood. The number of cells in each square should not vary more than 25 per cent. of the average number per square. The value of the count depends chiefly on the accuracy with which the blood is drawn up, and the thoroughness of the mixing. Chart result as the nearest multiple of 500.

Red Cell Counts: (Wood, p. 39). These are made similarly, using a 1:100 pipette, drawing the blood up to the 0.5 mark, or to 1.0 if patient is very anemic, and diluting to the 101 mark with Hayem's solution or 3 per cent. sodium chloride solution. The chamber is filled under the same precautions as in making the white counts, and 100 small squares counted in four blocks of 25 each. The sum of the cells counted is multiplied by 8,000 to obtain the number of cells per cubic mm. The counts of the different blocks should be within 20 per cent. of each other. A very common source of error is delay in putting the cover glass in position, resulting in counts that are too high. Chart result as the nearest multiple of 100,000. If Bürker's chamber is used the calculation is made as follows: For red cells at a dilution of 1:200 count all the cells in eighty of the smallest squares (each equivalent to 1/400 square mm.), point off two places, and the result will be the number of cells expressed in millions. For white cells at a dilution of 1:10 count one hundred of the larger squares, (each equivalent to 1/25 square mm.), and multiply by 0.025 to obtain the number of cells in thousands.

Counting Blood Platelets. A 1:100 dilution is made in a red cell pipette, from the second drop of blood emerging from a fresh puncture, the diluting fluid being freshly mixed as described in the section headed "Formulæ". Fill the counting chamber as usual but use a special thin cover so that the counting may be done with a high power dry objective. Allow the preparation to stand about ten minutes before counting so that the platelets will all have settled to the bottom of the cell. The red cells will appear as indistinct shadows, the leucocytes will show deep blue nuclei, and the platelets will appear as round or oval lilac colored bodies. Normally they number about 300,000 per cubic millimeter.

Oliver Hemoglobinometer. (One set of discs for this instrument is marked "Day light" and the other "Candle light", and only the corresponding type of illumination must be used.)

A large drop of blood is obtained, using special care not to squeeze out tissue fluids, and the tip of the small capillary tube

is touched to the drop. If the tube is clean and is held horizontally, it will fill promptly. The blood is then quickly wiped from the outside of the tip with finger, using care not to draw any blood out of the capillary, and leaving the column flush with the ends of the tube. The pipette is then filled with distilled water up to the 700 mm. mark (accurately) and the water blown through the capillary tube into the cell with the porcelain bottom so as to wash all the blood into the cell. It is mixed by stirring with the capillary tube and covered with the pale blue glass cover —forcing a drop into the metal chamber. The cell is then placed beside the scale so that the two are evenly lighted, and observed through the "telescope" holding one hole over the cell and the other over a disc on the scale. The scale is moved backward and forward until the disc which most nearly matches the diluted blood is obtained. The reading is made directly from the number above the scale disc. If the color is well between that of two of the discs, say 60 and 70, the percentage is considered as 65. Five per cent. is as close as one can expect to read.

Pink riders to be placed over the standard discs are provided for greater accuracy, but are of doubtful value. The darker rider, marked "5" adds 5 per cent. to the reading when placed over one of the standard discs of the upper half of the scale. The paler, marked "2.5" adds 2.5 per cent. to the upper six discs, and 5 per cent. to the lower discs. The capillary is cleaned by passing through it a needle threaded with soft cotton. The cell should be dried after using.

Fleischl-Miescher Hemoglobinometer (Wood, p. 26.) Blood is taken in the 1:200 mixing pipette to the 1.0 mark, and 0.1 per cent. sodium carbonate or distilled water is drawn in to the mark above the bulb. After slight shaking the pipette may be laid aside, but only for a short time, as the color of the solution rapidly changes. The 15 mm. cell is cleaned and dried, and the bottom gently screwed on. One side of the chamber is filled with distilled water. The first drops of the fluid are blown from the pipette, and the other side of the cell is then filled with the laked blood until a convex meniscus projects. The grooved cover slip is slid on so as not to include air bubbles and not to force the solution over the partition. The metal cap is put on with the slit at right angles to the partition. The cell is placed in the stage so that the "water" half lies over the glass wedge. The candle is

lit and the light adjusted; the dark box is covered and the reading is made through the tube. The wedge is slid forward and backward so as to make the near half of the field first too dark and then too light, until as nearly perfect a match as possible is obtained. Two independent readings should be made and averaged, and the nearest multiple of 5 used as the result per cent.

The accuracy of the readings can be greatly increased by proper use of the pipette. If the blood is drawn one sub-division beyond or short of the 1.0 mark, 1 per cent. is subtracted or added to the reading, as the case may be. Percentages below 30 are best determined by making a 1:100 dilution in an ordinary large bulb red blood counting pipette, and dividing the result by two. These irregular dilutions should be noted in writing when the blood is taken, to avoid confusion.

Cleaning Pipettes. Pipettes are cleaned by drawing through them, first, distilled water, then alcohol, then ether, then air. The bead should be freely movable without sticking to the side of the bulb, and the bulb should be dry and clean. If blood has dried in the pipette it should be removed with a horse hair or fine brass wire, such as is used for hypodermic needles, and the pipette then cleaned by filling with nitric acid or strong antiformin and allowing it to stand so over night. The Fleischl-Miescher cell should be taken apart, cleaned, and dried after use.

Blood smears for differential count or morphological examination are made on new slides. A differential count should always be made with a leucocyte count and a morphological examination with a red count. The slides are moistened with the breath and vigorously polished with a cloth. This gives them a slight warmth which is of advantage. A drop of blood is taken on the end of another slide as soon as it appears at the puncture, and is smeared by dragging, (or pushing), it across the first slide. The smearing slide is held at an angle of 45 degrees and should touch the first slide lightly. The smear is dried as *quickly as possible* by waving it in the air, or blowing on it and at the same time rubbing the back to warm it slightly. Smears for morphological examination or malaria hunts must be so thin that the red cells lie flat without touching each other. For differential counts they may be thicker, provided the leucocytes are flattened out enough to show their morphology clearly.

Fresh preparations of blood are made by taking a very small

drop of blood on the center of a cover slip, which has been cleaned very carefully in soap, water, and alcohol, and laying it on a slide similarly cleaned. If the glass is clean and the drop small, the blood will spread out, leaving the red cells separate. If the preparation is to be kept under observation for some time seal the edges of the cover glass to the slide with a little vaseline or cedar oil.

Jenner Stain. This is used for practically all purposes except malaria hunts. The slides are stood in a Coplin jar filled so as nearly to cover them with Jenner stain, and left three to five minutes. They are then removed, rinsed in one jar of distilled water, and washed in another, until of a lavender color, then blotted dry. The longer they are washed the more blue is removed and the pinker they appear. *Keep the Jenner jars covered.*

Wood Stain. Smears are fixed for three to five minutes in methyl alcohol, and blotted dry. They are then covered with 0.1 per cent. eosin for one minute; the excess of the stain is poured off, and a few drops of 0.25 per cent. methylene azure I are added for thirty to forty seconds. Longer treatment gives deeper stain. They are washed in distilled water and blotted dry. The stain can be made as desired by adjusting the length of time that each solution is allowed to act (Wood, p. 87).

Oxidase Reaction.

Fix the films by exposing to the vapor of strong formaldehyde under a bell jar for ten to fifteen minutes, or preferably use 4 per cent. formaldehyde for two to three hours. Dissolve a knife point of para-amido-dimethyl-aniline-sulphate in 2 c.c. of 1/10 normal NaOH solution and add an equal volume of 1 per cent. aqueous solution of alphanaphthol. Flood the specimen with this, apply a cover glass, and examine with the high power dry lens. All myeloid cells, that is, polynuclears, myelocytes, large mononuclears, transitionals, and myeloblasts will show blue granules, while lymphocytes and lymphoblasts will not. Compare a preparation made in this way with one stained with Jenner to form an idea of the relative proportion of cells giving and not giving the oxidase reaction.

Determining the Resistance of the Red Blood Cells.

This may be done on (a) unwashed, or (b) washed, cells, the former method being somewhat simpler. A series of accurately graduated salt solutions is prepared and kept ready for use. Weigh on a chemical balance 5 gm. of c. p. NaCl that has been dried in the oven at 120 C. for an hour or more, and make up to 500 c.c. with distilled water in a volumetric flask. From this 1 per cent. solution prepare a series of dilutions so that strengths ranging from 0.3 per cent. to 0.6 per cent. are obtained, varying by intervals of 0.025 per cent. Keep in *tightly stoppered* bottles.

(A). Unwashed cells. With a red cell counting pipette having a large bulb or Fleischl-Miescher hemoglobin pipette draw up blood to the 1 mark, and dilute with the salt solution of highest strength. Mix and expel the fluid completely into a small test tube. Make similar dilutions with each succeeding strength of salt solution. Place the rack of tubes in the incubator for an hour, or preferably in a water bath at 37 C. for an hour and then note in which tube hemolysis begins, as indicated by the appearance of a red tinge to the supernatant fluid, and in which tube hemolysis is complete, as shown by the total disappearance of the sediment of red cells.

(B). Washed cells. Collect about 5 c.c. of blood by venepuncture in 15 c.c. of 0.5 per cent. sodium citrate solution in 0.9 per cent. sodium chloride solution. Mix thoroughly and centrifugalize. Wash the cells twice with 0.7 per cent. sodium chloride solution and finally draw off the supernatant fluid as completely as possible. To make the test, measure exactly 1 c.c. of each of the hypotonic salt solutions into a series of test tubes and add to each 0.05 c.c. of the corpuscles, using a finely graduated pipette. Allow to stand one hour at 37 C. and read as above. With normal cells hemolysis begins at 0.425 and is complete at 0.350.

Vital Staining.

Puncture the finger and draw a good sized drop of blood into a red cell counting pipette, using the following solution, which must be freshly prepared, as a diluent:

Saturated solution of brilliant cresyl blue in 0.85 per cent. NaCl solution.....5 c.c.

Salt solution, 0.85 per cent.....5 c.c.
Sodium oxalate solution, 2 per cent.....2 c.c.
Add the oxalate to the salt solution, then mix with the staining fluid.

After thoroughly mixing the blood and stain in the pipette allow to stand for ten minutes, then blow the fluid into a centrifuge tube and centrifugalize. Draw off the supernatant fluid until only the sediment of cells remains. Take these up in a capillary pipette and smear on a slightly warmed slide. Allow to dry and examine with the oil immersion lens. (See Vogel and McCurdy, Archives of Internal Medicine, 1913, xiii, 707).

Coagulation Time.

This is determined at the bedside by means of the Boggs instrument. The instrument, microscope, Hagedorn needle, and watch are taken to the bedside. The truncated surface of the glass cone is carefully cleaned and dried, and a drop of water is placed in the bottom of the cell. The blood must be made to flow without much pressure; the first drop is wiped away, and the time that the second appears is noted. The tip of the cone is touched to the drop so that the truncated surface is just covered. The cone is then placed in the top of the cell, and the edge of the drop observed with a number 3 objective, shifting to different parts of the circumference. Air is then blown in, in small gentle puffs, by squeezing the rubber bulb or blowing. At first the cells move freely over each other, then in a mass in a direction parallel to the circumference and later are seen to snap back to their original position. Then, instead of moving in the direction of the circumference, they draw back toward the center of the drop and back toward the edge—radially. When this radial motion is first observed, the end time is noted, the cone is removed, and the drop is touched with a cloth or blotter to make sure of the presence of a coagulum. The time from the appearance of the drop on the finger to the appearance of radial motion is recorded as the coagulation time. A second test should always be made from a fresh puncture. The drop should not be blown upon too frequently or too vigorously. The time varies normally from three to eight minutes.

Hayem's Solution

Bichloride of mercury	0.5 gm.
Sodium sulphate	5.0 gm.
Sodium chloride	1.0 gm.
Aqua dist.	200 c.c.

Acetic Acid

Two per cent. solution of glacial acetic acid in distilled water; should be filtered every two or three days as a growth of moulds occurs quickly.

Platelet Counting Solution

Number I

Brilliant cresyl blue	1.0 gm.
Distilled water	300 c.c.

This solution is permanent but should be kept in the refrigerator to prevent the growth of moulds.

Number II

Potassium cyanide	1.0 gm.
Distilled water	1400 c.c.

This should be made every nine or ten days. When a count is to be made mix two parts of Number I with three parts of Number II immediately before use, and filter. If allowed to stand exposed to the air a precipitate will form in a short time.

Sodium Carbonate

0.1 per cent. solution in distilled water.

Jenner Stain

One-half gram of powdered stain is dissolved in 100 c.c. of pure methyl alcohol, allowed to stand for several hours, shaken at intervals, and then filtered. It must be kept well corked.

Aqueous Eosin

0.1 per cent. solution of Grübler "w.g." eosin in distilled water.

Methylene Azure

0.25 per cent. solution of methylene azure I in distilled water.

Pleural and peritoneal fluids. Routine examination consists in determination of specific gravity and amount of albumin, and a differential count of the cells.

Specific gravity is determined by a urinometer.

Albumin is determined by Esbach method. Two c.c. of fluid are measured with a pipette into 18 c.c. of distilled water in a graduate and mixed. An Esbach tube is filled to U mark with diluted fluid and to R mark with reagent; then inverted several times. After 24 hours the height of the sediment is read on the scale as grams of albumin to 100 c.c. of fluid.

Differential count is made after centrifuging fluid. The centrifuge tube is tilted and a little sediment is removed on to a warm slide and then spread with another slide like a blood film. It is stained with Jenner or Wood's stain. As a rule a hundred cells are counted, classifying as polynuclears and lymphocytes. The large endothelial cells present in most fluids should be disregarded or separately listed. Smears should be made as soon as possible while fluid is fresh. The cells degenerate as the fluid stands and become difficult to differentiate.

Spinal Fluid. As a routine a total count is made on the freshly shaken fluid and a differential from the centrifuged sediment.

The numerical count should be made as soon as possible after the fluid is drawn. The fluid is shaken gently and a drop placed in a counting chamber, or if bloody, a 1:10 leucocyte pipette is filled to the 0.5 mark with 30 per cent. acetic acid and fluid drawn up to the 11.0 mark, and the cells counted, using a number 6 objective. The dilution may be disregarded unless the count is high, when the total should be increased one-twentieth. Normal fluid contains about eight cells to the cubic millimeter.

Albumin may be roughly estimated by the following device. A narrow test tube about 5 mm. in diameter is strapped with adhesive to the side of an Esbach tube. Fluid is poured in to opposite the U mark, and Esbach's reagent to R mark. The readings are in grams to the liter.

Thick, ropy abdominal (cyst) *fluids* should be tested for mucin and pseudomucin (Wood, p. 651).

Milky, turbid, fluids should be examined for fat, etc., (Wood p. 636). For quantitative determination, see page 85.

Butyric Acid Test for Globulins. (*Noguchi*). To 0.2 c.c. of the spinal fluid (which must be free from blood) in a small test tube add 0.5 c.c. of 10 per cent. butyric acid solution in physiological salt solution, and boil for a few seconds. Add 0.1 c.c. of normal sodium hydrate solution and boil for a few seconds more. A positive reaction is shown by the appearance of a granular or floccular precipitate, which gradually settles to the bottom of the tube. In strongly positive fluids the precipitate appears in a few minutes, while an hour may be required in specimens containing only a little protein. Reactions occurring after two hours should be disregarded.

URINE.

Routine examination should determine transparency, color, reaction, specific gravity, and presence of albumin or sugar, and should include a microscopical examination.

Transparency. Make note on tag whether urine is turbid (Tr) or clear (Cl).

Color. Note only striking changes from normal pale yellow.

Reaction. Clip with scissors a small square of neutral litmus paper into each cylinder, and leave tag blank (if acid), or mark it N (neutral), or K (alkaline), according to whether the paper is turned red, is unchanged, or is blue, as compared with a slip moistened with distilled water.

Specific gravity. Make bob float freely in cylinder, not touching sides or bottom, read at the bottom of meniscus and mark the last two figures on the tag. If the quantity is insufficient, wait until the other tests have been done and then dilute the urine with an equal volume of water, or if the amount is still too small, with two volumes of water. Determine the specific gravity and multiply the decimal portion of the figure obtained by two, or three, according to the dilution used.

Albumin. Heat and acetic test. Fill row of test tubes in rack two-thirds full of urine, in the same order as specimens are arranged on the table. Add about 5 drops of 2 per cent. acetic acid to each tube, or enough to make the reaction acid, and boil each tube at the top, holding tube at the bottom and directing the flame against the upper portion of the fluid. Add a few more drops of the acid and then examine the tubes by transmitted light against a black background for a cloud in top portion as compared with portion just below it. If there is no clear portion, filter another sample, using Kieselguhr if necessary, acidify and boil, before reporting it negative. If precipitate is flocculent, take tube in holder and heat entire contents to boiling and stand tube in rack above the specimen glass, marking tag "%". When precipitate has settled, fifteen minutes or more afterward, mark the percentage according to the estimated proportion of the column of urine occupied by the sediment. Precipitates too small to settle out are recorded on tag as

"V. f. tr." (very faint trace)

"F. tr." (faint trace)
"Tr." (trace)
"M. tr." (marked trace), or
"V. m. tr." (very marked trace) according to the intensity of the cloud. If the precipitate is indefinite, "V. f. tr.?" may be used, and a sulphosalicylic acid or Spiegler's test made.

Sulphosalicylic Acid Test. Use in cases where heat test is doubtful, or when it is negative and casts are found in the sediment. Stratify urine over 20 per cent. sulphosalicylic acid and look for white ring, or better, add a little of the acid powder to a small tube of urine and boil. Then compare with a control sample in the same size tube and note any cloudiness.

Spiegler's Test. (May be used in same cases). Acidify *strongly* with *glacial* acetic acid and stratify urine over Spiegler's reagent. An opaque white ring appearing immediately indicates an abnormal amount of albumin. Normal urine will show a ring on standing.

Sugar. Fehling's Test. With pipette with rubber nipple put about 1 c.c. of urine in a row of test tubes in the order in which the specimens are arranged on the table, putting three specimens in each test tube, so that the first tube will represent specimens one to three, the second, four to six, the sixth, sixteen to eighteen, etc. Add to each tube about 5 c.c. of boiling Fehling's solution from casserole, and boil each tube, replacing it in the rack. If a red, yellow, or yellowish green precipitate is formed, test the three corresponding specimens separately. If a typical red or golden yellow precipitate is formed, mark tag "sugar"; if only a greenish yellow is obtained, do a phenylhydrazin test, as follows:

Phenylhydrazin Test. Five drops of phenylhydrazin are mixed in a test tube with ten drops of glacial acetic acid and one c.c. of saturated solution of sodium chloride; two or three c.c. of urine are added, and the tube is heated for at least two minutes over the flame. The test tube is allowed to cool for a few minutes, and the crystals which settle to the bottom are removed with a pipette and examined under the microscope.

Benedict's Test. This is more sensitive than Fehling's and also less open to error since the reagent is not reduced by uric acid, creatinine, chloroform, or the simple aldehydes. Into each of a series of numbered test tubes in a rack place five c.c. of the

reagent and eight drops of each of the urines in numerical order. Immerse the rack in a water bath filled with boiling water and keep the water boiling. At the end of exactly five minutes remove the rack and allow the tubes to cool. A positive reaction is shown by the fluid becoming opaque. If no sugar is present the fluid remains clear or only a faint turbidity results due to urates. Large quantities of albumin should first be removed.

Indican. To one-third test tube of urine add an equal volume of Obermayer's reagent, shake, and add 2 c.c. of chloroform. Allow to stand several minutes with occasional shaking. The indican is reported as

"F. tr." (faint trace)
"Tr." (trace)
"M. tr." (marked trace)
"+", "++", and "+++", according to the depth of the blue color.

Blood. Guaiac Test. To about 4 c.c. of urine add 1 c.c. of glacial acid and 2 c.c. of ether; shake gently, pour off the ether, and add a few drops of freshly prepared guaiac tincture and 1 c.c. of hydrogen peroxide. Never use a test tube with yellow copper oxide on its walls resulting from Fehling's or Benedict's sugar tests.

Benzidine Test. Dissolve a knife point of Merck's "Benzidine for Blood Testing" in 2 to 3 c.c. of glacial acetic acid, warming if necessary. Add 10 to 15 drops of the fluid to be tested and then add 3 per cent. peroxide of hydrogen solution drop by drop until a blue or green color appears or 2 c.c. have been added.

Diazo Reaction. Mix 50 c.c. of diazo reagent I with 1 c.c. of diazo reagent II and put about 10 c.c. of the urine with about 10 c.c. of this mixture in a test tube; add 15 to 20 drops of ammonia and shake. A positive reaction is shown by a deep cherry red color in the fluid, and a bright pink shade in the foam. Yellow or orange colors are negative.

N. B. Typhoid urines often contain virulent bacilli.

Acetone. Drop a crystal of sodium nitroprusside in 5 to 10 c.c. of water. Add 1 to 2 c.c. of this solution and a few drops of glacial acetic acid to 5 c.c. of urine, and stratify strong ammonia over the mixture. A purple ring at the junction of the fluids constitutes a positive reaction.

Diacetic Acid. Add a few drops of 10 per cent. ferric chloride

to about 10 c.c. of urine, drop by drop. If a precipitate forms, filter, and add a few more drops of ferric chloride. A "Burgundy" red constitutes a positive reaction. If the patient has been taking phenol, salicylates, aspirin, acetanilid or antipyrin, a dark color will also be produced on the addition of ferric chloride, but it will be a purple rather than a red. In such cases dilute 20 c.c. of the urine with an equal amount of water, boil in a beaker or porcelain dish until the volume has been reduced to the original amount of urine taken and repeat the test. The color due to diacetic acid will be much fainter or will not appear at all, while that due to drugs will not be affected.

Arnold's Test. This is more delicate than the Gerhardt test above, and is not interfered with by drug reactions. Two parts of Arnold's reagent number I and one part of reagent number II are mixed together in a test tube, and an equal bulk of urine is added. Strong ammonia is added, drop by drop. This gives a brown color. An excess of hydrochloric acid is then added, which in normal urine changes the brown to yellow, but in the presence of diacetic acid produces a purple color and on shaking a violet foam.

Bile. 1. Shake urine in test tube to produce foam. A yellow tinge to foam indicates bile. If the acid urine is filtered and fuming nitric acid dropped on the yellow filter paper, a green ring will form about the acid if bile is present. The acidified urine may also be layered over the acid.

2. Layer a very dilute alcoholic solution of iodine over urine. A green ring shows bile. The iodine solution should be of a pale yellow color.

Urobilin. To a test tube containing urine add 25 drops of 10 per cent. zinc chloride and enough ammonia to dissolve the bulk of the precipitate. Filter off the remaining precipitate (phosphates) and hold the test tube against a dark background and in a ray of bright light, such as that of an electric pocket lamp. An excess of urobilin is indicated by a band of green in the fluid.

Urobilin may also be detected by direct examination with a spectroscope. The characteristic band is one between the green and the blue parts of the spectrum. The absorption band is sharper if a few drops of iodine are added to 10 c.c. of urine. The band is marked only when an excess of urobilin is present.

Do Quantitative Tests only with Twenty-four Hour Specimens

Quantitative Sugar Determination. Take 25 to 100 c.c. of a twenty-four hour specimen, acidify slightly with acetic acid, and add about 5 grams of powdered normal lead acetate. Filter through dry filter paper. Pour the perfectly clear filtrate into clean tube of saccharimeter till there is a convex meniscus above the top. Slide on cover slip without including air bubble, and screw on cap. Adjust instrument so as to throw daylight through tube. Focus eyepiece so that disc is sharp. Determine zero point without the tube by rotating the analyzer slightly, making the right half disc darker, and then lighter than the left, until the two colors match. Make three readings and average. Insert tube, make three readings, and average, correcting for zero if necessary. The result is per cent. sugar, if rotation is clockwise. Multiply by twenty-four hour amount to obtain grams per day. Do not attempt to read smaller amounts than 0.5 per cent. Oxybutyric acid, if present in large amounts, is sufficiently levorotatory to render polariscopic reading inaccurate. In such cases either use Benedict's quantitative method (see chemical section) or ferment out sugar with yeast, clear, and estimate levorotation. Add this to the reading obtained before fermentation.

To read vernier: I. To the right of O. Read division on primary scale to left of O on the vernier, which is the unit reading. Count to right on vernier scale until line is found which is exactly opposite a line on the primary scale. The number of this vernier line in tenths, (that is, divided by 10) is added to the unit reading obtained from the main scale.

First reading 3; vernier reading 7; result 3.7.

II. To the left of O. Read in same way, but subtract the vernier reading from primary reading.

First reading 3; vernier reading 7; result 2.3.

Quantitative Albumin Determination. Esbach Test. Fill Esbach tube with urine to U mark and with Esbach reagent to R mark. Invert several times so as to mix without shaking and remove any foam with a strip of filter paper. Read in twenty-four hours. The scale shows tenths of 1 per cent. If the albu-

min on boiling is 20 per cent. or more, dilute urine ten times before doing Esbach test.

Total Urea. Doremus Method. Take 15 c.c. of 20 per cent. NaOH and add 35 c.c. of water and 1 c.c. of bromine. Fill Doremus tube and introduce 1 c.c. of urine with special pipette into closed arm of tube, injecting slowly so that bubbles do not escape from bulb. Allow to stand ten minutes and read on scale (milligrams per c.c.). Multiply the result by number of c.c. in twenty-four hour specimen and divide by 1000 to obtain grams per day.

Normal amount, 20 to 30 grams.

The method is extremely inaccurate.

Microscopical Examination.

Place a drop obtained with a pipette from bottom of each specimen glass (or from top layer of urate or pus sediment) on glass plate in order in which the specimens are arranged. Rinse pipette between each specimen to avoid carrying over sediment. Examine each drop carefully under number 3 objective for casts—hyaline. granular, or waxy ("h.c.", "gr.c.", "wax.c."), for cells—epithelial ("ep"), leucocytes ("l"), or red blood cells ("r.b.c."), and for crystals or amorphous deposits—uric acid ("ur. ac."), urates ("ur."), ammonium urate ("am. ur."), amorphous phosphates ("phos."), triple phosphate ("3 phos."), dicalcium phosphate ("ca. phos."), calcium oxalate ("ca. ox."), or other rarer sediments.

Do not report casts without a following "?" unless both ends can be clearly made out to be rounded and fairly abrupt, not tapering. Unless granules are numerous and on focusing appear in the substance of the cast, report cast as "hyaline".

The occurrence of true casts without albumin is rare, and large quantities of albumin rarely occur without either casts. blood, or pus.

The amount of pus is indicated by stating whether it forms a definite sediment or merely a cloud through the specimen, or is only microscopically visible. The presence of even a few polynuclear leucocytes should be reported as pus cells.

SPIEGLER'S REAGENT

Mercuric chloride	80 gm.
Tartaric acid	40 gm.
Glycerine	200 c.c.
Water	2000 c.c.

BENEDICT'S QUALITATIVE SOLUTION

Copper sulphate (cryst.)	17.3 gm.
Sodium or potassium citrate	173.0 gm.
Sodium carbonate (cryst.)*	200.0 gm.
Distilled water to make	1000.0 c.c.

The copper sulphate dissolved in about 100 c.c of water is poured slowly, stirring constantly, into the citrate and carbonate previously dissolved in about 700 c.c. of hot water. The mixture is cooled and diluted to 1 liter.

FEHLING'S ALKALINE SOLUTION

Sodium and potassium tartrate	346 gm.
Sodium hydrate	100 gm.
Water	1000 c.c.

FEHLING'S COPPER SOLUTION.

Copper sulphate (cryst.)	69.278 gm.
Distilled water to make	1000. c.c.

OBERMAYER'S REAGENT

Conc. hydrochloric acid (sp. gr. 1.19) containing 2 gm. of ferric chloride to the liter.

EHRLICH'S DIAZO REAGENT. NUMBER I

Sulphanilic acid	1 gm.
Hydrochloric acid (sp. gr. 1.19)	50 c.c.
Water	1000 c.c.

EHRLICH'S DIAZO REAGENT. NUMBER II

Sodium nitrite	1 gm.
Water	200 c.c.

ESBACH'S REAGENT

Picric acid	10 gm.
Citric acid	20 gm.
Water	1000 c.c.

* One-half this amount of the anhydrous salt may be used.

ARNOLD'S REAGENT, SOLUTION NUMBER I

Para-amido-aceto-phenone	1 gm.
Water	100 c.c.
Hydrochloric acid (conc.)	2 c.c.

ARNOLD'S REAGENT, SOLUTION NUMBER II

Potassium nitrite	1 gm.
Water	100 c.c.

EHRLICH'S ALDEHYDE REAGENT

Para-dimethyl-amido-benzaldehyde	15 gm.
Water	270 c.c.
Sulphuric acid (conc.)	30 c.c.

GASTRIC CONTENTS AND STOOLS.

Test Meals. Ewald meals should be sent to the laboratory as soon as expressed. The amount of free acid should be determined immediately; the rest of the examination may be deferred. If a delay is necessary, the specimen should be put on ice. An analysis includes tests for:

1 Total acidity
2 Free hydrochloric acid
3 Combined hydrochloric acid (if free is present)
4 Lactic acid (if free HCl is absent or very low)
5 Blood
6 Rennin
7 Bile.

The contents of the cup are measured and note is made of the color, odor, condition of the bread (whether in fragments as chewed or well macerated) and amount of mucus present (whether well mixed with the meal). Anything unusual in the appearance of the specimen is noted, such as the presence of excess of water, or of undigested egg or other unexpected articles of diet. A drop of sediment is examined under the microscope for food elements, blood cells, pus, yeasts, sarcinæ, and Boas-Oppler bacilli (coarse Gram-positive bacilli, motile, and occurring in chains; very numerous when present). The meal is then carefully strained through two layers of gauze placed in a funnel.

1. *Total acidity.* Ten c.c. are carefully measured with a pipette into a flat porcelain dish, and two or three drops of phenolphthalein added. Decinormal sodium hydrate is then added from

a burette until the red color when spread evenly does not become deeper on addition of another drop. The number of c.c. used is multiplied by 10 and the result used to express the acidity. The usual normal reading is between 40 and 60, but 80 is not abnormal.

2. *Free hydrochloric acid.* Amido-benzol paper is touched with the fluid. If it turns red, free mineral acid is present.

Ten c.c. of the strained juice are measured into another porcelain dish with a pipette and a drop of Töpfer's reagent (dimethyl-amido-azo-benzol) is added. Decinormal soda is then added until the orange-red color changes to a bright yellow—the end reaction. The result is again expressed in the number of c.c. which would be required to neutralize 100 c.c. of gastric contents. The normal amount is given as from 20 to 60, with an average of 40.

If the fluid is small in amount, the free HCl should be determined first, and then a few drops of phenolphthalein added to the same sample and titration continued to determine the total acidity. The amount of alkali used in neutralizing to Töpfer's reagent must of course be included in this reading. If necessary 5 c.c. or even less may be used for titration, but this increases the error.

3. *Combined hydrochloric acid.* This determination can not be made unless there is free HCl present. As an indicator alizarin is used, which reacts to free acid, and to acid phosphates, but not to the loosely combined acid to which phenolphthalein is also sensitive. Ten c.c. of juice with a few drops of alizarin solution are titrated with decinormal alkali. The color changes from yellow to red and then to violet. The end reaction is a violet which is not deepened by the addition of another drop of alkali. The number of c.c. used is subtracted from the number required to neutralize the juice to phenolphthalein, and the difference, multiplied by 10, used to indicate the amount of acid in combination with proteid. Normal readings are from 0.5 to 15 c.c.

4. *Lactic acid.* Specimens with a low free acidity should be tested for lactic acid.

Kelling's Test. Add a few drops of $FeCl_3$ solution to a test tube of distilled water sufficient to produce a very faint yellow. Divide this into two portions and to one add a few drops of the

juice. Lactic acid will produce a distinct "canary yellow" in the tube to which it is added.

5. *Blood.* If brown, questionable particles are seen in the sediment, these should be selected for the test. If not, the fluid should be tested as described under urine. Take 3 c.c. of fluid, 3 c.c. of glacial acetic acid, and 2 c.c. of ether; shake and allow to separate. If large amounts of fat are present extract with neutral ether first. Add 5 to 10 drops of fresh guaiac tincture and 2 c.c. of H_2O_2. A blue color in the ether indicates blood. Benzidine may also be used as described under urine.

6. *Rennin.* Take a few drops of the test meal and add 10 c.c. of milk. If HCl is absent or low in amount mix 1 drop of 1 per cent. calcium chloride solution with the gastric juice before adding it to the milk.

7. *Bile.* A yellow juice may be tested for bile by layering under alcoholic iodine as for urine; a green juice by layering over yellow nitric acid, when a red color may appear from the further oxidation of the biliverdin; but, as a rule, inspection is sufficient.

Vomitus. Examined only as ordered on the tag. The complete routine examination is not necessary.

Stools.

Stools are examined as requested on the tag. For *microscopical examination,* a small particle is taken on platinum loop or glass stirring rod, and, after mixing on the slide with a drop of water, if the stool is solid, is spread in a thin layer under a large cover glass, exerting no pressure. Ova are best found by searching the thin areas with the low power, but it is always advisable to examine a portion of the drop with high power. Two or three drops from different portions of the stool should be examined before a negative report is made.

Muscle fibers are usually bile stained and the preservation of striations is an index of their lack of digestion. Fatty acids occur in sheaves of delicate needles, and fat as refractile droplets which may be identified by adding Scharlach R. Calcium soaps may appear as round bile stained masses. Starch is tested for by running iodine solution under the slide.

Stools for amebas should be sent to the laboratory while still warm and fresh, and examined immediately on the warm stage. While that is being set up they are kept in the incubator. Parti-

cles of bloody mucus should be examined if any can be found. Then smears are made and examined with number 6 objective. *Entameba histolytica* varies greatly in size; it is motile, possesses a distinct ectosarc, and as a rule contains red blood cells. It measures from 30 to 70 micra while *Ameba coli* is usually less than 30 micra.

Stools to be searched for *gall stones, worms,* etc., are placed in gauze, or better, in a fine sieve under a cold water tap in some remote spot. They are washed with occasional stirring and rubbing with a rod or spatula until all soluble and granular portions are washed away. The residue of coarse vegetable fibers, etc., must then be searched.

Blood is tested for by the guaiac or benzidine test (see Urine and Gastric Contents). Solid stools are best rubbed up with a very little water before adding the acetic acid. It should be remembered that meat in the food and iron salts given as medication, will frequently give a positive reaction. The stools should first be extracted with neutral ether; the extract thrown out and the residue treated with acetic acid, extracted and tested in the usual way with guaiac or benzidine.

Bilirubin and *hydrobilirubin* are detected by Schmidt's test. A small amount of stool is placed in a porcelain dish and thoroughly mixed with about an equal volume of saturated solution of bichloride of mercury and allowed to stand for six hours or more. Particles containing hydrobilirubin turn pink or red; those containing bilirubin turn green (biliverdin).

Formulæ.

Phenolphthalein: 1 per cent. solution in 95 per cent. alcohol.

Di-methyl-amido-azo-benzol: 0.5 per cent. solution in 95 per cent. alcohol.

Alizarin: 1 per cent. solution of alizarin S in water; filtered.

Ferric chloride: 5 per cent. solution.

CLINICAL ANALYTICAL METHODS

USE OF THE BALANCE

See that the balance is perfectly level, as indicated by the plumb bob; if it is not, make it so by turning the adjusting screws at the right and left front corners.

Always place the object to be weighed on the left-hand pan, and the weights on the right-hand pan. One reason for this is to avoid errors through possible inequalities in the length of the two arms of the beam. To obtain the true weight of a substance when extreme accuracy is required, counterbalance it with sand or weights and then replace the object with weights; or weigh in one pan and then exchange the substance and weights, weigh again, and take the mean of the weights so obtained.

The beam and pans must always be supported before adding or removing weights, and the weights must be handled only with forceps.

To avoid errors in noting weights always count them twice: 1st, by noting those missing from the box; 2d, by noting the weights as they are taken from the pan and replaced in the box

All objects must be at room temperature when weighed. Warm objects can not be weighed accurately as currents of air are caused which introduce an error.

A platinum crucible should remain in the desiccator ten to fifteen minutes, and a porcelain crucible twenty to twenty-five minutes before weighing.

The supports of beam and pans must be lowered gently to avoid injury to the knife edges.

The balance case must never be left open or with the beam unsupported, and the rider must be removed from the beam.

Be careful to avoid spilling the substance to be weighed on the pans or on the floor of the balance case. If this happens, remove at once by dusting carefully with a camel's hair brush.

Liquids must be weighed in closed weighing bottles, and solids in weighing bottles or watch glasses.

From time to time determine the zero point of the scale. Set the pans swinging by producing a current of air against them by moving the hand palm down briskly downward in front of one pan, and then close the door of the case. Designate the central division of the scale as 10, and determine the points on the scale to which the pointer moves on either side after the first two or three swings. Take at least two readings on one side and one on the other, and obtain the mean; e.g.:

Left	Right
5.6	13.6
6.0	
2)11.6	13.6
5.8	13.6

$5.8+13.6=19.4$

$$\frac{19.4}{2} = 9.7 = \text{zero point}$$

To determine the tenths of a milligram, either use a rider or, if great accuracy be required, estimate them by interpolation, as follows:

Weigh to the nearest milligram too light, and obtain the zero point as described above. Call this reading Y. Add 1 milligram and again obtain the zero point, calling this Y^1. Call the zero point of the balance unloaded A. Then the fraction of a milligram to be added to the weight of the object will be equal to

$$\frac{Y-A}{Y-Y^1}$$

VOLUMETRIC METHODS.

Burettes: With colorless solutions, read the bottom of the meniscus. With colored solutions, such as permanganate, read the top of the column of fluid. To avoid errors through parallax keep the eye at the level of the top of the column of fluid. Solutions should always be at the standard temperature engraved on the volumetric apparatus when they are measured.

Keep burettes covered with caps when not in use, and always use a funnel to fill the burette, but remove it before adjusting the level of the fluid.

As the absolute error of reading remains constant, the percentage of error is greater the smaller the amount of solution used; and the quantity of liquid employed should, therefore, preferably not be less than 20 to 25 c.c.

Burettes and pipettes must be free from grease or the solution will not moisten the surface of the glass evenly and will collect in droplets. To clean burettes and pipettes, use a mixture of 100 c.c. concentrated sulphuric acid to which 1 gram of potassium bichromate has been added. Fill the burettes with the mixture, allow to stand for some time, and then wash carefully in water. Finish by rinsing with distilled water.

Pipettes are ordinarily made to discharge the amount indicated by the graduation; measuring flasks to contain it. In both instances the bottom of the meniscus is the point read.

The opening of the pipette should be small, so that from ten to twenty seconds will be required to empty the instrument. Allow the point to touch the side of the receiving vessel as the last drops flow out.

The upper end of the pipette and the finger used in closing it must be dry in order to control the flow of liquid properly.

If corrosive fluids are to be measured with a pipette, always slip a piece of rubber tubing 20 to 30 cm. in length over the end of the instrument to avoid the possibility of getting fluid into the mouth. Never draw ammonia into a pipette with the mouth.

INDICATORS.

These are bases, acids, or salts, which change color in the presence of acids and alkalies respectively, and possess so intense a color as to enable small amounts to tinge relatively large quantities of fluid.

The indicator itself takes part in the reaction between the acid and alkali; that is, salts are formed or are decomposed into their respective bases and acid radicles. It is this alteration in chem ical constitution that gives rise to the change in color.

The indicators in most common use and their leading characteristics are as follows:

Indicator	Color with Acids	Color with Alkalies	Use with Carbonic Acid in Cold Sol.	Use with Carbonic Acid in Hot Sol.	Use With Ammonium Salts	Use with Organic Acids
Litmus	Red	Blue	Un*	Re.	Re.	Re.
Methyl orange	Pink	Yellow	Re.†	Un.	Re.	Un.
Phenolphthalein	Colorless	Pink	Un.	Re.	Un.	Un.
Cochineal	Purple-red	Blue	Re.	Re.	Re.	Re.
Alizarin red	Yellow	Red	Un.	Re.	Re.	Re.
Congo red	Blue	Red	Un.	Re.	Re.	Un.

STANDARD SOLUTIONS.

As ordinarily employed, the term "normal solution of an acid" is used to indicate a solution of such a concentration as to contain 1 gram of replaceable hydrogen per liter; e.g. normal HCl solution contains the molecular weight of HCl (36.458) per liter; but normal H_2SO_4 contains only one-half the molecular weight, because H_2SO_4 has two replaceable H atoms. Similarly, the concentration of a normal solution of an alkali is such that the amount of the base present corresponds to the amount of acid in the normal acid solution; e.g., normal NaOH contains 40.058 gm. of NaOH per liter, this being its molecular weight, but of sodium carbonate only one-half the molecular weight would be taken, as one molecule of this base is capable of replacing two atoms of acid hydrogen.

Since oxygen (atomic weight 16) is bivalent, oxidizing solutions are made on the basis of 8 grams of available oxygen per liter. For example, in potassium permanganate ($KMnO_4$, molecular weight 158.15) five atoms of oxygen of the eight contained in two molecules of the salt are available for oxidizing purposes; i.e., 80 parts by weight; therefore, one-tenth of this double molecule, (2 x 158.15) or 31.63 grams must be taken for the normal solution, and 3.163 for the decinormal solution.

PREPARATION OF N/10 SULPHURIC ACID.

Take of Merck's anhydrous reagent sodium carbonate about 7 to 8 gm. and ignite gently in a previously weighed platinum crucible, not allowing the heating to exceed a dull red in order to avoid the conversion of small amounts of carbonate into

*Unreliable. †Reliable.

hydroxide, which may take place at high temperatures. The object of the heating is to dehydrate the salt completely and to decompose any bicarbonate which may be present.

Allow to cool in the desiccator, and on the balance quickly remove enough to leave exactly 5.3 gm. in the crucible.

Dissolve this in hot distilled water, rinsing the crucible well. Allow the fluid to cool and then make up to exactly one liter.

Take 7 c.c. of chemically pure H_2SO_4 (sp. gr. 1.84), dilute with four or five volumes of distilled water, and allow to cool.

Transfer to a two liter Giles' flask, and add distilled water to the 2200 c.c. mark. Shake well, and fill a 50 c.c. burette with the acid, and another with the sodium carbonate solution, in each case rinsing out the burette first with some of the solution.

Measure 50 c.c. of the carbonate into a beaker, add a few drops of methyl orange, and titrate with the acid until a pink tinge is noticeable and the addition of a drop of alkali restores the neutral color. Repeat until duplicates are obtained differing by not more than 0.1 c.c.

The acid will be found to be too strong and the amount of water to be added is poured into the flask after reducing the quantity to exactly 2 liters. The amount of water is calculated as follows: for example,

49.5 c.c. of acid neutralizes 50 c.c. of the alkali; then

$$C = \frac{N \cdot d}{n} \qquad C = \frac{2{,}000 \times .5}{49.5} \qquad C = 20.2$$

C = No. of c.c. of water to be added.
N = Amount of solution remaining.
d = Difference between no. of c.c. theoretically required and no. of c.c. actually used.
n = No. of c.c. used in titration.

Repeat the titration and correction until the two solutions are adjusted so as to balance evenly. If the acid is too weak, it is simpler to make it a little too strong again by adding a drop or two of concentrated acid, and then diluting to the required degree.

PREPARATION OF N/10 SODIUM HYDRATE.

Weigh out about 10 gm. of pure NaOH*. Dissolve in water, and dilute to 2200 c.c. in a Giles' flask.

*Merck's reagent sodium hydroxide purified by alcohol is satisfactory.

Standardize with the N/10 H_2SO_4 in the manner described above. For work in which Congo red is to be used habitually it is advisable to employ this indicator in adjusting the alkali to the acid.

Keep in a rubber stoppered bottle.

MELTING POINT DETERMINATION.

Draw a piece of glass tubing into a capillary having a bore of 0.75 to 1. mm.; cut off a portion of this 8 cm. in length; and seal one end. Introduce a small quantity of the substance to be tested by pressing the open end of the tube against the powder and then tapping it down or pushing it in with a fine wire. Attach it to the thermometer with a small rubber band, in such a way that the powder is at the level of the bulb of the thermometer, and the rubber band is as high up as possible. Immerse the bulb of the thermometer in concentrated sulphuric acid in a test tube, which is then heated in sulphuric acid in a beaker of Jena glass or, preferably, in the special Thiele apparatus, and heat with a small Bunsen flame. Watch the substance and note the temperature at which it suddenly becomes transparent or contracts into a droplet. The heating should be slow. If a beaker is used, stirring is necessary to equalize the temperature. At least three determinations should be made.

A somewhat simpler method which is available for many substances is to fill with mercury a porcelain dish about 10 to 12 cm. in diameter. A thermometer is suspended over this in such a way that the bulb is completely immersed in the mercury but does not touch the bottom of the dish. Several particles of the substance under investigation are dropped on the surface of the mercury near the thermometer bulb and the dish is slowly heated with a flame. Watch for the melting point as above.

To correct the temperature for the error due to the exposed portion of the mercury thread, use the following formula:

$S = T + n\ (T - t) \times 0.000159.$

S = Corrected melting point.

T = Observed melting point.

n = Length of exposed portion of thread in degrees.

t = Temperature of air surrounding the exposed air as determined by another thermometer. Ordinarily the room temperature may be used.

Clean the glass float and platinum wire, by immersing for a moment in concentrated sulphuric acid. Rinse with water, and dry carefully with filter paper.

Fill the cylinder with urine, and bring this to the required temperature of 15 C. by immersing the cylinder in water of a suitable temperature. Adjust the balance with the leveling screw at the foot so that it is in equilibrium with the float hanging in air.

Immerse the float in the cylinder of urine, adjusting the height of the balance so that the float is completely covered, but only a little of the platinum wire dips below the surface of the fluid when the beam is horizontal. Hang the first rider on the same hook as the float, and then bring the balance into equilibrium by hanging other riders on the various notches. The riders on the beam give the specific gravity to four decimals, the integers following in the order of the size of the riders and being indicated by the numbers of the respective notches.

PYCNOMETER.

Weigh the pycnometer empty, and then fill with distilled water at 15 C, drying the outside carefully with filter paper, and weigh again. Weigh again filled with urine at 15 C. Deduct the weight of the instrument in each case, and obtain the specific gravity by dividing the weight of the urine by that of the water.

If only very small amounts of fluid are available, as is sometimes the case in dealing with specimens of urine obtained by catheterizing the ureters a fairly accurate determination of the specific gravity may be made as follows: Into a stoppered weighing bottle of which the weight is known measure with a finely graduated pipette one, two, three, or more c.c. of the fluid in question which is at room temperature, and weigh. Repeat the process with the same amount of distilled water which is also at room temperature. Divide the weight of the urine by the weight of the water. A red cell blood counting pipette may also be used as a pycnometer.

DETERMINATION OF THE NITROGEN PARTITION IN THE URINE.

The chief nitrogenous constituents of the urine are urea, uric acid, ammonia, and creatinine. Under normal conditions these

together make up about 95 per cent. of the total nitrogen of the urine, the balance being composed of small amounts of creatine, amino acids, hippuric acid, allantoin, purin bases, pigments, etc. As carried out for clinical purposes, a complete nitrogen partition includes the determination of the total N and of the amounts of urea, uric acid, ammonia, and creatinine, together with a statement of their nitrogen content, and the percentage which this is of the total N. Ordinarily, however, the factors of greatest interest to the practitioner are the total N, the urea N, and the ammonia N.

Accurately collected twenty-four hour specimens are essential for quantitative determinations on the urine. As a preservative about 10 c.c. of toluol or 5 per cent. alcoholic solution of thymol per liter of urine may be used, and the receptacle should be kept in a cold place. The calculations ordinarily may be simplified by diluting the entire specimen with water to the nearest round number of c.c., as 1000, 1500, etc.

TOTAL NITROGEN DETERMINATION IN URINE.
KJELDAHL METHOD.

Principle: By digestion with concentrated sulphuric acid in the presence of an oxidizing agent, all nitrogenous constituents in the organs, fluids, or excretions of the body are converted into ammonia and the organic bodies decomposed. Potassium sulphate facilitates the reaction by raising the boiling point of the acid. The ammonia is bound by the acid and forms ammonium sulphate. This is then liberated by the addition of alkali and is distilled into a measured amount of decinormal acid. The amount of acid so neutralized may then be determined by titration with decinormal alkali.

Reagents: Concentrated sulphuric acid.
Potassium sulphate.
$CuSO_4$.
30% NaOH.
N/10 H_2SO_4.
N/10 NaOH.
Congo red.
Pumice.

Technique: Measure 5 c.c. of urine with a graduated pipette into a Kjeldahl flask of 800 c.c. capacity. Add 10 c.c. of H_2SO_4

and a small crystal of copper sulphate, and heat over wire gauze in a fume closet until white fumes are given off. Then add a couple of grams of potassium sulphate and continue the heating until the fluid has lost all yellow color, and then for about one-half hour longer. Allow the flask to cool, and carefully and slowly dilute the contents with distilled water to about 400 c.c. Add about a teaspoonful of powdered pumice, and for each 10 c.c. of sulphuric acid used, add 60 c.c. of 30% sodium hydrate without shaking, and avoiding contact of the alkali with the mouth of the flask, as otherwise it is difficult to make the rubber stopper stick tightly. The flask is immediately connected with the safety bulb of the distilling apparatus. An Erlenmeyer flask.* of 500 c.c. capacity has previously been prepared with 50 c.c. of decinormal H_2SO_4, 10 drops of Congo red, and a little distilled water, and adjusted under the condenser so that the tip of the glass safety tube of the latter dips below the surface of the fluid. The distillation is started (at first with a small flame) and continued for at least an hour. The drops falling from the condenser are then tested with litmus paper. If still alkaline, the distillation is continued. Otherwise the receiving flask is lowered, the distillation continued for a few minutes longer, and the flame extinguished. The safety tube is detached and is rinsed with distilled water inside and out into the receiving flask. The fluid is then titrated with N/10 NaOH, the end reaction being a brilliant red. In dealing with untested reagents a blank determination is necessary to determine their possible nitrogen content, and this amount should be deducted from the result.

Each c.c. of N/10 acid neutralized = 0.0017 gm. of NH_3 and 0.0014 gm. of N.

The usual amount of nitrogen on an average diet is 15 to 16 gm. in 24 hours.

UREA DETERMINATION IN URINE.

1. BENEDICT.

Principle: In the presence of potassium bisulphate and in the absence of water, urea is hydrolyzed at 160 C. almost quantitatively into ammonia and carbon dioxide in about an hour, zinc sulphate being added to make the decomposition of the urea complete. Under these conditions uric acid and creatinine are

*Quart milk bottles are cheaper and very satisfactory for this purpose.

not hydrolyzed. The potassium bisulphate, having a replaceable H atom, retains the ammonia, which is then liberated by the addition of alkali and distilled into a known amount of standard acid.

Reagents: Potassium bisulphate.
Zinc sulphate.
Powdered talcum.
30% NaOH.
N/10 H_2SO_4.
N/10 NaOH.
Congo Red.

Technique: Place 5 c.c. of urine (which must not contain sugar) in a wide test tube (preferably provided with an ampulla near the top), add 3 grams of potassium bisulphate, 1.5 grams of zinc sulphate and a small quantity of talcum. Evaporate almost to dryness, either cautiously over a flame or by immersion in a bath of sulphuric acid or paraffin at about 120 C. If the mixture foams badly add a fragment of paraffin the size of a pea. After dryness has been reached, heat the tube in the bath at 162 to 165 C. for one hour. The tube is removed and the contents washed into an 800 c.c. Kjeldahl distillation flask and diluted to 400 to 500 c.c. Add a little talcum or pumice and 10 c.c. of 30% NaOH solution. Distill into 50 c.c. of N/10 H_2SO_4 and titrate as in the Kjeldahl determination (q.v.).

Result: Each c.c. of N/10 NH_3 equals 0.0014 gm. of N, or 3 mgm. of urea. The amount of urea may also be obtained by multiplying the amount of urea N by the factor 2.14. Before calculating the amount of urea N and urea, the N of the preformed ammonia must be determined and deducted.

2. VAN SLYKE AND CULLEN.

Principle: Urease, an enzyme obtained from the soy bean, converts urea into ammonium carbonate. It acts at room temperature, but is most active at a temperature of about 55 C. and in a perfectly neutral solution. It decomposes nothing but urea, and is not interfered with by the presence of glucose. The urea in the urine is converted into ammonium carbonate by the enzyme, the ammonia in the latter is then liberated by the addition of alkali, and is aspirated into a measured

amount of standard acid. The ammonium carbonate formed would by its alkaline reaction retard the action of the enzyme, but the development of alkalinity is prevented by the presence of potassium acid phosphate (KH_2PO_4). An excess of this, however, interferes with the combination of enzyme and urea which precedes hydrolysis of the latter so that it is advisable to use the minimum amount of phosphate that will keep the reaction sufficiently near the neutral point.

Reagents: Soy bean enzyme (Urease)*

0.6% solution of acid potassium phosphate, (KH_2PO_4)

N/50 H_2SO_4

N/50 NaOH

Potassium carbonate solution, 90 grams to 100 c.c. water

Caprylic alcohol†

Alizarin

Technique: Place 5 c.c. of urine in a 50 c.c. volumetric flask and dilute to volume. Pipet 5 c.c. of this diluted urine (equal to 0.5 c.c. of the original) into a wide mouthed test tube, add a soy bean tablet (previously crushed to powder) or 1 c.c. of freshly made enzyme solution, 5 c.c. of 0.6% KH_2PO_4 solution and 2 drops of caprylic alcohol. Connect the tube with the receiving tube prepared as below and allow to stand for 15 to 20 minutes.

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*Either in tablets of 0.1 gm. each containing also 5% of neutral potassium phosphate (K_2HPO_4) to aid in preserving the activity of the solution, or in solution freshly made as follows: solid enzyme, one part by weight in ten of water. First mix to a paste with a little water, then add the rest of the water in portions, forming a cloudy solution.

Standardization of Enzyme. Urease as supplied by the manufacturers varies somewhat in activity, and fresh supplies must always be tested in the following way:

Test solution:

Di-basic potassium phosphate (K_2HPO_4)	43 gm.
Diacid potassium phosphate (KH_2PO_4)	34 gm.
Urea	60 gm.
Water to make	1000 c.c.

Place 5 c.c. of this solution in the tube of the aeration apparatus and bring to exactly 20 C. in a water bath. Add 1 c.c. of 10 per cent. enzyme solution also at 20 C. and allow to stand at this temperature for exactly 15 minutes. Add 6 or 7 grams of potassium carbonate and aerate as usual into 50 c.c. of N/50 acid. The result is expressed in the number of c.c. of N/50 acid neutralized. This should be about 40.

† An efficient antifoaming mixture which is much cheaper than caprylic alcohol may be made as follows:

Diphenyl oxide	60 c.c.
Amyl alcohol	40 c.c.

Into the receiving tube of the aeration apparatus place 25 c.c. of N/50 H_2SO_4 and 2 drops of alizarin. At the end of the 15 minutes allow the air current to pass for half a minute to remove any ammonia that may be free in the urine tube, and then open the latter and introduce 5 c.c. of potassium carbonate solution. Close the tube at once and aerate, first slowly and then more rapidly for fifteen minutes to half an hour, according to the strength of the suction. Then titrate with N/50 NaOH.

Result: The difference between the number of c.c. of acid in the receiver and the amount of alkali needed for neutralization equals the number of c.c. of N/50 NH_3 yielded by the quantity of urine taken. From this result the preformed ammonia (separately determined) must be deducted. Each c.c. of N/50 NH_3 equals 0.00028 of N, and the urea nitrogen multiplied by the factor 2.14 gives the amount of urea. Or, the number of c.c. of acid neutralized by the ammonia from the urine multiplied by 0.056 equals the grams of urea N plus ammonia N per 100 c.c. of urine.

The urea on an average diet is 20 to 40 grams per 24 hours, and equals about 85 to 90 per cent. of the total N.

AMMONIA DETERMINATION IN URINE.

Principle: The ammonia is liberated by the addition of a weak alkali, is removed from the urine by the passage of a strong current of air, and is collected in N/50 sulphuric acid. The amount of the latter neutralized by the ammonia is then determined by titration with N/50 alkali.

Reagents: N/50 H_2SO_4.
N/50 NaOH.
Potassium carbonate solution, 90 grams to 100 c.c. of water.
Alizarin.
Petroleum.

Technique: Use the same apparatus as that employed for the determination of the urea by the urease method. In the receiving cylinder place 25 c.c. of N/50 H_2SO_4 and 3 drops of alizarin. In the aerating cylinder place 5 c.c. of urine, 5 c.c. of potassium carbonate solution, and 1 c.c. of petroleum. Allow the air current to pass first slowly and then more rapidly for about an hour in all. Then titrate back with N/50 NaOH.

Result: Each c.c. of N/50 H_2SO_4 neutralized by the ammonia

liberated corresponds to 0.00028 of N, or 0.00034 of NH_3. In normal urine the average amount of ammonia is about 0.7 grams in 24 hours, or 4 per cent. of the total N.

URIC ACID DETERMINATION IN URINE.
COLORIMETRIC METHOD.

Principle: The uric acid is precipitated by the use of ammoniacal silver solution, the supernatant fluid is discarded, and the precipitate is redissolved with potassium cyanide. The solution is then treated with the phosphotungstic reagent which gives a blue color proportional in depth to the amount of uric acid present; this is compared in the colorimeter with the color produced by a standard solution of uric acid under similar conditions.

Reagents: Ammoniacal silver magnesia mixture.

3% silver lactate ..... 70 c.c.
Magnesia mixture* ..... 30 c.c.
Concentrated aqueous ammonia 100 c.c.
5% Potassium cyanide solution.

Uric Acid Reagent.

Sodium tungstate .....100 gm.
Phosphoric acid, 85%..... 80 c.c.
Water .....750 c.c
Boil for one hour in a flask with a funnel in the neck, cool, and dilute to one liter.

20% Sodium Carbonate Solution.

Uric acid standard solution:

Disodium hydrogen phosphate (cryst.) 9 gm.
Sodium dihydrogen phosphate (cryst.) 1 gm.
Hot water 200 to 300 c.c. If not clear, filter and make up to about 500 c.c. While still hot pour in 200 mgm. of uric acid suspended in a little water in a liter flask. Shake till the uric acid is dissolved, and allow to cool. Add exactly 1.4 c.c. of glacial acetic acid, shake, dilute

*Magnesia mixture is made as follows:
Crystallized magnesium sulphate ..... 17.5 gm.
Ammonium chloride ..... 35. gm.
Concentrated ammonia ..... 60. c.c.
Water to make .....200. c.c.

to the mark, and add about 5 c.c. of chloroform to prevent the growth of moulds. Five c.c. of this solution equals exactly 1 mgm. of uric acid.

Technique: From 2 to 4 c.c. of urine, according to the amount of uric acid expected, is measured into a centrifuge tube, diluted to about 5 c.c. with water and treated with 15 to 20 drops of ammoniacal silver solution. Mix thoroughly with a small stirring rod and allow to stand for ten minutes. Centrifuge, and then pour off the supernatant fluid as completely as possible, inverting the tube over a piece of filter paper. Aspirate the ammonia vapors from the tube by suction with the filter pump and then add to the residue in the tube 2 drops of 5% potassium cyanide solution. Stir well, add 10 to 15 drops of water and stir again. Add 2 c.c. of uric acid reagent, stir, add 10 c.c. of 20% sodium carbonate solution. Allow to stand about half a minute and wash quantitatively into a 50 c.c. graduated cylinder. According to the depth of color as compared with the standard it is diluted to 25 or 50 c.c. The standard is prepared simultaneously by treating 5 c.c. of the standard uric acid solution in a 50 c.c. cylinder with two drops of potassium cyanide solution, 2 c.c. of uric acid reagent, and 10 c.c. of 20% sodium carbonate solution. Dilute to 50 c.c. at the end of a half minute and compare the two in the Duboscq colorimeter.

Result: The calculation is made as follows:

$$X = \frac{S}{R} \times 1 \text{ mg.} \times \frac{Rd}{Sd} \times \frac{Y}{W}$$

X = Mg. of uric acid in 24 hour amount of urine.
S = Reading of Standard.
R = Reading of Unknown.
Rd = Dilution of Unknown.
Sd = Dilution of Standard.
Y = 24 hour volume of urine in c.c.
W = Amount of urine in c.c. taken for determination.

1 mgm. of uric acid equals 0.000333 gm. of N. The amount of uric acid usually present is 0.2 to 1.5 gm. in 24 hours, equivalent to 0.5 to 2.5% of the total N.

TITRATION METHOD.

Principle: The uric acid is precipitated as ammonium urate which is then decomposed by the addition of H_2SO_4; and the liberated uric acid is titrated with N/20 potassium permanganate solution. To remove the mucoid substances always present, which would interfere with the subsequent operations, the urine is first treated with a reagent containing ammonium sulphate and uranium acetate. The resulting precipitate of uranium phosphate carries down the mucoid body. Ammonia is then added to alkalinize the solution and cause the precipitation of the ammonium urate.

Reagents: Solution of uranium acetate, made as follows:

Uranium acetate,	5 gm.
Ammonium sulphate,	500 gm.
Acetic acid, 10%,	60 c.c.
Water,	650 c.c.

Strong ammonia.
Ammonium sulphate solution, 10%.
N/20 potassium permanganate solution.
Strong sulphuric acid.

Technique: Into a tall beaker or cylinder measure 200 c.c. of urine and 50 c.c. of the uranium reagent. Allow to stand for one-half hour, and then decant, siphon, or filter off the supernatant fluid. Measure 125 c.c. (equal to 100 c.c. of urine) of this into a beaker, add 5 c.c. of strong ammonia, and set aside until the following day. Filter off the precipitate and wash with 10% ammonium sulphate solution until the filtrate is nearly or quite free from chlorides. Remove filter from funnel, open and wash the precipitate into a beaker with the ammonium sulphate solution. Add water to make 100 c.c. and dissolve the precipitate with 15 c.c. concentrated sulphuric acid. Titrate at once with the N/20 potassium permanganate solution. The end reaction is the first pink coloration extending through the entire liquid from the addition of two drops of permanganate solution while stirring with a glass rod, and remaining for 30 seconds.

Result: Each c.c. of permanganate solution used corresponds to 3.75 mgm. of uric acid; add 3 mgm. as correction due to solubility of ammonium urate.

1 mgm. of uric acid = 0.000333 gm. of N.

The amount usually present is 0.2 to 1.5 gm. in 24 hours; that is, 0.5 to 2.5% of the total nitrogen.

CREATININE DETERMINATION IN URINE.

Principle: On adding picric acid and sodium hydrate to a solution containing creatinine, a deep red color is produced. The intensity of this in the specimen of urine is compared with that of a standard solution of potassium bichromate. Sugar and albumin do not interfere; but acetone and diacetic acid, if present, must be removed by heating.

Reagents: N/2 potassium bichromate solution (24.55 gm. per liter)
Sat. picric acid solution (about 12 gm. per liter)
10% NaOH.

Technique: Ten c.c. of urine is measured into a flask with a mark at 500 c.c., 15 c.c. of picric acid solution and 5 c.c. of sodium hydrate are added, and the mixture allowed to stand for five minutes. Pour a little bichromate solution into the two cylinders of the Duboscq colorimeter and set the left hand one to the 8 mm. mark. Then make several readings in order to accustom the eye to the colors. Now dilute the urine mixture to the 500 c.c. mark, and rinse out and half fill one of the cylinders with it, wipe off the glass rod, and then make several readings immediately and take the average. The reading must be made within ten minutes.

If the urine contains more than 15 mgm. or less than 5 mgm. of creatinine, repeat the determination with a smaller or larger amount of urine, as outside of these limits the determination is less accurate.

Calculation: $10 \times \frac{8.1}{\text{Reading}} = \text{mgm. creatinine.}$

1 mgm. creatinine = 0.000371 gm. N.

The normal excretion of creatinine is about 20 to 30 mgm. per kilo of body weight, fat persons yielding less and thin persons more. On an average diet the creatinine nitrogen equals about 3 to 5 per cent. of the total nitrogen.

CREATINE DETERMINATION IN URINE.

Principle: On heating creatine with dilute mineral acids it is dehydrated and its anhydride creatinine is formed. At a temperature of 117 to 120 C. the conversion is complete in fifteen minutes. This temperature is reached when the pressure is one kilo per square centimeter, or 14 pounds per square inch.

Technique: Place 20 c.c. of the urine in a 500 c.c. Erlenmeyer flask, add the same amount of normal hydrochloric acid, and heat in the autoclave for twenty to thirty minutes at 117 to 120 C. Cool and make the volume up to exactly 50 c.c. with distilled water, shake thoroughly, and measure off 25 c.c. of the mixture, corresponding to 10 c.c. of the original urine. Neutralize this with 10 c.c. normal sodium hydrate solution, and then determine the creatinine by the method described above. From the amount of creatinine so obtained deduct the amount of creatinine determined in the unheated urine. The difference will be the creatine content of the original urine in terms of creatinine. To obtain the amount of creatine multiply this figure by the factor 1.16. The dark color produced by the heating usually causes no difficulty, owing to the dilution necessary in making the mixture for the colorimeter.

MISCELLANEOUS DETERMINATIONS.

DETERMINATION OF CHLORIDES IN URINE.

Principle: The chlorides in a definite amount of urine are precipitated by a standard solution of silver nitrate in the presence of an excess of free nitric acid. The precipitate of silver chloride is filtered off, and the excess of silver remaining in solution is determined in the filtrate by titrating with a standard solution of potassium sulphocyanide, using a solution of iron alum as an indicator. As soon as the sulphocyanide has combined with all the silver to form white silver sulphocyanide, the deep red ferric sulphocyanide is formed and indicates the end reaction. In this way the amount of silver solution which combined with the chlorides is ascertained. Albumin need not be removed unless present in large amount.

Reagents: $AgNO_3$ solution, of which 1 c.c. equals 0.01 NaCl.*

Potassium sulphocyanide solution, of which 1 c.c. equals 1 c.c. of the $AgNO_3$ solution.

Saturated solution of ammonioferric alum.

Nitric acid.

Technique: Ten c.c. of urine, accurately measured with a pipette, is placed in a flask with a mark at 100 c.c.; about 50 c.c. of water, 5 c.c. of nitric acid, and 20 c.c. of the silver solution are added. The nitric acid is to be measured in a graduate, but the silver solution must be accurately measured.

The mixture is well shaken, and distilled water is added to the 100 c.c. mark. The fluid is filtered through a small dry filter, and 50 c.c. taken, this amount corresponding to 5 c.c. of urine. This 50 c.c. is then poured into a beaker and the measuring flask rinsed into the same with distilled water.

Five c.c. of the alum solution is added, and the whole is titrated with the sulphocyanide solution to the appearance of the first reddish tinge.

Calculation: 20 minus the no. of c.c. of sulphocyanide solution used equals the no. of c.c. of silver solution required to precipitate the chlorides. Each c.c. of silver solution equals 0.01 of NaCl; therefore, 20 minus twice the reading, x 10 = milligrams of NaCl in 10 c.c. of urine. The usual amount in 24 hours is 10 to 15 grams.

DETERMINATION OF PHOSPHATES IN URINE.

Principle: The urine is first treated with a solution of sodium acetate and glacial acetic acid in order to convert any monacid phosphate into diacid phosphate, and also to neutralize any nitric

*The silver nitrate solution should contain 29.059 gm. of $AgNo_3$ per liter, and the solution of potassium sulphocyanide equivalent to this requires 16.62 gm. per liter. As the latter salt is hygroscopic, the exact amount cannot be weighed accurately, so weigh out about 22 gm. and dissolve in 1200 c.c. of water. Pipette 10 c.c. of the silver solution into a porcelain dish, dilute with 100 c.c. of distilled water, acidify with nitric acid and add 5 c.c. of ammonioferric alum solution. Titrate with the sulphocyanide solution to the appearance of a permanent reddish color and then dilute the sulphocyanide solution to the proper degree according to the principles given on page 44.

To check the accuracy of the silver solution, dry chemically pure sodium chloride at 120 C. and weigh out exactly 0.150 gm. Dissolve in 100 c.c. of distilled water, add a few drops of 5 per cent. potassium chromate solution, and titrate with the silver solution till an orange tint appears. Exactly 15 c.c. of the silver solution should be necessary.

acid that may be formed during the subsequent titration, as this, if allowed to remain free, would cause partial solution of the precipitated uranyl phosphate. The titration is then performed with a standard solution of uranium nitrate which gives with phosphoric acid compounds in acetic acid solution a yellowish white precipitate of uranyl phosphate (UO_2HPO_4). As indicator cochineal is used, which gives a green color in the presence of an excess of uranium solution. Sugar and albumin do not interfere with the method. If the urine is deeply bile stained, it should be acidified with HCl and decolorized by the addition of a few crystals of potassium permanganate.

Reagents: A solution of uranium nitrate of which 20 c.c. equals 0.1 gm. P_2O_5.

Acetic acid solution: Sodium acetate, 100 gm.
Glacial acetic acid, 30 gm.
Water to 1000 c.c.

Tincture of cochineal.

Technique: Fifty c.c. of filtered urine is treated with 5 c.c. of the acetic acid mixture. A few drops of the cochineal indicator are added. The fluid is heated to boiling and titrated with the uranium solution until a trace of green color is seen, which does not disappear on stirring. The titration is repeated until accurately corresponding duplicates are obtained, and the result is calculated as follows:

20 : 0.1 : : n : x, n being the number of c.c. of uranium solution used.

The percentage of P_2O_5 equals 2x.

The usual amount of P_2O_5 on an average diet equals 1 to 5 gm. in twenty-four hours.

DETERMINATION OF SULPHATES IN URINE.

Sulphur occurs in the urine in the following forms:

1 Inorganic or preformed sulphates.
2 Ethereal or conjugated sulphates in which H_2SO_4 is combined with aromatic compounds.
3 Neutral or unoxidized sulphur.

The partition of the three forms is determined as follows. The urine must be freed from albumin.

BENZIDINE METHOD.

Principle: The sulphates are precipitated as an insoluble salt of benzidine (p-diaminodiphenyl, $NH_2 . C_6H_4 . C_6H_4 . NH_2$) and this is then dissociated by the addition of a volumetric solution of sodium hydrate.

Reagents: Benzidine solution.*
Dilute HCl (1 part concentrated HCl to 4 parts H_2O by volume)
Water saturated with benzidine sulphate†.
N/10 NaOH.
Phenolphthalein.

INORGANIC SULPHATES.

Technique: Into a 250 c.c. Erlenmeyer flask pipette 25 c.c. of urine and add dilute HCl until it is distinctly acid to Congo red paper (usually 1 to 2 c.c.). Add 100 c.c. of the benzidine solution and allow to stand for ten minutes. The precipitate is filtered off by suction using a small Büchner funnel or a funnel with a perforated porcelain filter plate, or with a platinum filtering cone. Do not allow the precipitate to be sucked dry. Wash the precipitate with water saturated with benzidine sulphate until the filtrate gives no reaction with Congo red (10 to 20 c.c.). Transfer precipitate and paper back to the original flask with about 50 c.c. of water and titrate hot with N/10 NaOH and phenolphthalein until pink.

Result: Multiply the number of c.c. of N/10 alkali used by

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*Rub into a paste with about 10 c.c. of H_2O 4 grams of Kahlbaum's or Merck's benzidine. Transfer this paste with 500 c.c. of H_2O to a two liter volumetric flask, add 5 c.c. of concentrated HCl (Sp. gr. 1.19) and shake until dissolved. Finally dilute to 2000 c.c. 150 c.c. of this solution, which keeps indefinitely, precipitates 0.1 gm. of H_2SO_4.

†**Preparation of Benzidine Sulphate.** 18.4 gm. of p-benzidine are dissolved in 50 c.c. of alcohol, filtering if solution is not complete. 5.7 gm. of concentrated (95%) sulphuric acid (sp. gr., 1.84) measured in a graduated pipette and also dissolved in 50 c.c. of warm alcohol is slowly added to the benzidine solution with constant stirring. When the precipitate has settled, test the supernatant alcohol with moistened blue litmus paper, adding a little more alcoholic acid if necessary to insure a slight acidity. The grayish white benzidine sulphate is then filtered on a Büchner funnel, using suction, washed with alcohol several times, once with ether, and dried on a water bath. If the precipitate appears to be at all lumpy, it is best to grind it thoroughly in a porcelain mortar before filtering and washing.
Yield, 28 gm.

.004 to obtain the number of gm. of SO_3 present in 25 c.c. of urine.

TOTAL SULPHATES.

Principle: The ethereal sulphates are split by boiling with HCl and the total sulphates resulting are determined just as above.

Technique: Twenty-five c.c. of urine and twenty c.c. of dilute HCl are gently boiled in an Erlenmeyer flask for 15 to 20 minutes. Cool, neutralize with sodium hydrate, then make acid to Congo red with dilute HCl and proceed as above.

ETHEREAL SULPHATES.

The amount of these is obtained by subtracting the amount of the inorganic sulphate from that of the total sulphate.

TOTAL SULPHUR.

Reagents: Those used above.

Benedict's solution:

Crystallized copper nitrate,	200 gm.
Sodium or potassium chlorate,	50 gm.
Distilled water to	1000 c.c.

Principle: All of the sulphur present is oxidized by heating with a reagent composed of copper nitrate and potassium chlorate. The former on heating decomposes into two vigorous oxidizing agents: nitrogen dioxide and cupric oxide, the latter forming a stable compound with the oxidized sulphur. This is dissolved in dilute hydrochloric acid, and the sulphur precipitated with benzidine as above.

Technique: Ten c.c. of urine is measured into a small (7 to 8 cm.) porcelain evaporating dish, and 5 c.c. of the reagent added. The contents of the dish are evaporated over a free flame, which is regulated to keep the solution just below the boiling point, so that there can be no loss through spattering. When dryness is reached the flame is raised slightly until the entire residue has blackened. The flame is then turned up in two stages to the full heat of the Bunsen burner and the contents of the dish thus *heated to redness for ten minutes after the black residue* (which first fuses) *has become dry.* This heating is to decompose the last traces of nitrate and chlorate. The flame is then removed and the dish allowed to cool more or less completely, 10 to 20

c.c. of dilute (1:4) HCl is next added to the residue in the dish, which is then warmed gently until the contents have completely dissolved and a perfectly clear, sparkling solution is obtained. This dissolving of the residue requires scarcely two minutes. With the aid of a stirring rod the solution is washed into a small Erlenmeyer flask, is neutralized with sodium hydrate, made acid to Congo red with dilute HCl, and then precipitated with benzidine as above.

The total sulphur per 24 hours usually varies from 1 to 3.5 gm. of SO_3. Of this the inorganic sulphates are about 85 to 90%; the ethereal sulphates, 8 to 10%; and the neutral sulphur about 5%, but the sulphur partition is largely modified by changes in diet.

BARIUM CHLORIDE METHOD.

Principle: The sulphates in the urine are precipitated by the addition of an excess of barium chloride solution. The precipitate of barium sulphate is filtered off, washed, dried, ignited and weighed.

Reagents: Dilute HCl (1 part concentrated HCl to 4 parts H_2O by volume)
Barium chloride solution, 5 per cent.

INORGANIC SULPHATES.

Technique: Into an Erlenmeyer flask place about 100 c.c. of water; 10 c.c. of dilute hydrochloric acid, and 25 c.c. of urine. If the urine is dilute take 50 c.c. instead of 25, and a correspondingly smaller amount of water. Ten c.c. of barium chloride solution is added drop by drop from a pipette having a short piece of rubber tubing slipped over its upper end and provided with a screw pinchcock. The urine must not be disturbed while the barium chloride is being added. At the end of an hour or later, the mixture is shaken and filtered through a weighed Gooch crucible, as described below. The precipitate is washed with at least 200 c.c. of water. The crucible is then dried, ignited, placed in the desiccator, cooled, and then weighed.

The increase in weight indicates the amount of $BaSO_4$ referable to the inorganic sulphates in the amount of urine used.

1 gm. $BaSO_4$ = 0.34301 gm. SO_3.
1 gm. " = 0.42015 gm. H_2SO_4.
1 gm. " = 0.13744 gm. S.

To express the results in terms of SO_3, H_2SO_4, or S, multiply the weight of $BaSO_4$ by the proper factors as given above.

TOTAL SULPHATES.

Principle: The ethereal sulphates are split by boiling with HCl, and the total sulphates resulting determined just as above.

Technique: Twenty-five c.c. of urine and 20 c.c. of dilute HCl (or 50 c.c. of urine and 4 c.c. of concentrated HCl) are gently boiled for 20 to 30 minutes in an Erlenmeyer flask, into which a funnel has been placed to reduce the loss of steam. The flask is cooled for about 2 to 3 minutes in running water, and the contents are diluted with cold water to about 150 c.c. Then precipitate with barium chloride, filter through a Gooch, and weigh as above.

ETHEREAL SULPHATES.

The amount of these may be obtained by subtracting the amount of inorganic sulphates from that of the total sulphates.

TOTAL SULPHUR.

Oxidize the sulphur with Benedict's sulphur reagent as described under the benzidine method. The solution obtained by dissolving the residue in the porcelain dish is washed quantitatively into a small Erlenmeyer flask, diluted with cold distilled water to 100 to 150 c.c., 10 c.c. of 5 per cent. barium chloride solution is added drop by drop and the solution is allowed to stand for about an hour. It is then shaken up and filtered as usual through a weighed Gooch filter.

To make the Gooch crucible filter, pour a suspension of asbestos fiber in water into the crucible while strong suction is being applied, so that a firm felt work (about 2 mm. thick) is formed. The asbestos is prepared by scraping the crude material with a knife and adding the fibers to a large bulk of 5% HCl in a cylinder. Air is blown through to separate the fibers thoroughly, and the mixture is allowed to settle for a few minutes. The upper portion of the fluid containing the finer fibers is decanted and kept separately from the lower.

In making the filter the coarse material is poured on first and a little of the fine afterward. The filter is then washed by drawing distilled water through in a slow stream, is dried at 120 C., ignited, and weighed.

In igniting the barium sulphate precipitates the flame must not be applied directly to the bottom of the crucible, in order to avoid mechanical losses. The crucible is placed on a platinum crucible lid on a pipe stem triangle, and the outer, oxidizing flame of the Bunsen burner is used first gently and finally with full force.

The results obtained by these procedures will be in terms of barium sulphate. It is customary to report them in terms of SO_3. This is done by multiplying the amount of $BaSO_4$ by the factor 0.34301.

TOTAL ACIDITY DETERMINATION IN URINE.

Principle: Determination of the acidity of the urine by direct titration is not possible owing to the occurrence of calcium and ammonium salts in the presence of monobasic phosphates. The addition of potassium oxalate, however, overcomes these difficulties by holding in solution the di- and tricalcium phosphates and preventing dissociation of the ammonium compounds.

Technique: To 25 c.c. of urine add 15 to 20 gm. of powdered potassium oxalate and several drops of phenolphthalein.

Shake for several minutes, and titrate at once with N/10 sodium hydrate until a distinct permanent pink is obtained, shaking the flask during the titration.

To neutralize a 24-hour amount of urine from 550 to 650 c.c. of N/10 alkali are usually required.

FREEING URINE FROM ALBUMIN AND THE GRAVIMETRIC DETERMINATION OF ALBUMIN.

Take 100 c.c. of urine. If necessary make it faintly acid with dilute acetic acid, and heat on the water bath until the albumin begins to separate in flakes. After drying the outside of the beaker boil for two minutes over a free flame.

If the albumin does not coagulate well, carefully add a drop or two of dilute acetic acid. Excess of acid may cause some of the albumin to stay in solution.

Filter while still hot through a Schleicher and Schüll ash-free filter (No. 589, 9 cm.) that has previously been dried at 120 C. and weighed. If further quantitative determinations are to be made on the urine, filter into a measuring flask and wash out the beaker and filter with small amounts of distilled water until the volume at room temperature is brought up to its original

amount. Wash precipitate on the filter with more warm water and then with alcohol followed by ether.

Dry in the hot air oven at 120 C. to constant weight.

If great accuracy be desired, fold the filter carefully with the precipitate inside, wrap a platinum wire about it, hold it over a weighed platinum crucible and burn the filter paper, allowing all the ash to fall into the crucible. Then ignite, weigh again, and take the weight of the ash from the weight of the dried substance.

The albumin may also be removed before doing a nitrogen partition by adding an equal volume of alumina cream* and filtering.

DETERMINATION OF GLUCOSE IN URINE.

BENEDICT.

Principle: Through the substitution of sodium carbonate for the strong alkali in Fehling's solution, Benedict's solution is made much more sensitive as a reagent for glucose. The reduced copper produced when the solution is boiled in the presence of glucose is precipitated not as the red suboxid (Cu_2O), but as the white cuprous sulphocyanate (CuSCN) which makes it easy to determine when the last trace of blue has been removed from the solution, showing complete reduction of all the copper.

Reagents: Benedict's quantitative solution†
Sodium carbonate, crystallized.
Powdered pumice.

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*The aluminum hydroxide for this purpose is made as follows: To a 1 per cent. solution of ammonium alum add a slight excess of 1 per cent. solution ammonium hydroxide at room temperature. Wash the precipitate by decantation until the wash water gives a very faint residue on evaporation (Tracy and Welker).

† Benedict's quantitative solution is made as follows:

	gm. or c.c.
Copper sulphate (pure crystallized)	18.0
Sodium carbonate (crystallized)	200.0
Sodium or potassium citrate	200.0
Potassium sulphocyanate	125.0
5% potassium ferrocyanide solution	5.0
Distilled water to make a total volume of	1000.0

With the aid of heat dissolve the carbonate, citrate, and sulphocyanate in enough water to make about 800 c.c. of the mixture, and filter if necessary. Dissolve the copper sulphate separately in about 100 c.c. of water and pour the solution slowly into the other liquid, with constant stirring. Add the ferrocyanide solution, cool and dilute to exactly 1 liter. Of the various constituents, the copper salt only need be weighed with exactness. Twenty-five c.c. of the reagent are reduced by 50 mgm. of glucose.

Technique: Twenty-five c.c. of the reagent is measured with a pipette into a porcelain evaporating dish, and 10 to 20 gm. of crystallized sodium carbonate or one-half the weight of anhydrous sodium carbonate are added, together with a little pumice stone or talcum. The mixture is heated over a free flame until the carbonate is dissolved and then the urine, diluted 1:10, (unless the sugar content is expected to be very low) is run in from a burette rather rapidly until the entire disappearance of the blue color, which marks the end point. During the entire titration the fluid must be kept boiling vigorously and the water lost by evaporation may be replaced from time to time.

Result: If the urine was diluted 1:10, the percentage of sugar may be obtained as follows:

$$\frac{0.050}{X} \times 1000 = \text{percentage of sugar in undiluted urine, X being the number of c.c. of diluted urine required to produce the end reaction.}$$

DETERMINATION OF ACETONE AND DIACETIC ACID IN URINE.

Principle: The pre-formed acetone and that developed from diacetic acid by heating is aspirated into a known amount of hypoiodite solution. The iodine unites with the acetone to form iodoform, and by titration with thiosulphate solution the amount of the former remaining uncombined is ascertained. By subtracting this from the amount originally taken the amount entering into combination with the acetone is learned and the quantity of the latter may be determined by calculation.

Reagents: N/10 Iodine solution*.
N/10 Sodium thiosulphate solution†.
40% potassium hydroxide solution.
10% phosphoric acid.
Sodium chloride.
Kerosene.
Starch solution (1% solution of soluble starch with one drop of cinnamic aldehyde per 100 c.c.)

*N/10 iodine solution contains 12.692 gm. iodine to 1000 c.c. To make it dissolve in a liter flask 20 gm. of pure potassium iodide in a little water. Add 12.8 gm. of sublimed iodine and shake gently until dissolved. Dilute to 1000 c.c. If necessary the solution may be filtered through asbestos. Standardize against N/10 sodium thiosulphate solution by titrating 25 c.c.

Technique: Into the receiver of the aeration apparatus introduce 150 c.c. of water, 10 c.c. of 40% potassium hydroxide solution and exactly 50 c.c. of N/10 iodine solution. Use a wide test tube as an aeration cylinder and arrange this so that heat may be applied during aeration. Into the tube place 20 c.c. of the urine to be examined, 10 drops of 10% phosphoric acid, 10 gm. of sodium chloride (acetone is insoluble in saturated salt solution) and a little kerosene to prevent foaming. It is well to attach a wash bottle containing hypoiodite solution to the other side of the aeration tube in order to free the in-coming air from possible traces of substances capable of combining with the iodine. The apparatus is connected with a pump and air is allowed to pass for half an hour or more, according to the strength of the air current. During this period the urine mixture is heated just to the boiling point and after an interval of five minutes is again heated in the same manner. The fluid in the receiving cylinder is acidified with concentrated hydrochloric acid, an excess of iodine solution being shown by the development of a brown color. Add a little starch solution and titrate with N/10 sodium thiosulphate until the blue color disappears. The end point may be determined more accurately if the starch is not added until the titration is almost finished, as shown by the brown color becoming paler.

Result: X = (A-Y) x 0.967.

A = number of c.c. of iodine solution in receiving cylinder.

Y = number of c.c. of thiosulphate solution used in titrating.

X = mg. of acetone (pre-formed and that from diacetic acid) in the amount of urine used.

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of iodine solution with the thiosulphate until the yellow color of the solution is just discharged by one drop of thiosulphate.

†N/10 sodium thiosulphate solution contains 24.824 gm. of the salt ($Na_2S_2O_3$·5 H_2O) to 1000 c.c. To make it dissolve 30 gm. of the crystalline salt in 1000 c.c. of water. Standardize with potassium acid iodate as follows; Dissolve in a little water exactly 0.1625 gm. of pure potassium acid iodate. Add about 1 gm. of potassium iodide and a few c.c. of dilute HCl. After a few minutes titrate with the thiosulphate solution so that the final yellow color of the fluid is just discharged by one drop of the thiosulphate solution. This is then diluted so that the volume consumed in titration shall measure exactly 50 c.c.

One c.c. of N/10 iodine solution is equivalent to 0.967 mgm. of acetone.

"The oxidation method, combined with the preliminary distillation for the removal and determination of acetone and diacetic acid, is carried out as follows: From 25 to 100 c.c. or more of urine (usually 50 c.c.) are measured with a pipette into a 500 c.c. volumetric flask containing 200 c.c. to 300 c.c. of water. Basic lead acetate solution (U. S. P.) is added in amount equal to the urine used* and the liquid well mixed. Strong ammonia water, about half the volume of the lead acetate, is next poured in the flask, diluted to the mark with water, shaken, and after a few minutes' standing, the liquid is filtered, preferably through a folded filter, 200 c.c. of the filtrate is measured into a round bottom flask (800 c.c. or liter Kjeldahls are convenient) diluted with water to about 600 c.c.. 15 c.c. of concentrated sulphuric acid and talc or boiling stone added, and the mixture distilled until about 200 c.c. of the distillate have collected (Distillate A).

"The distilling flask must be fitted with a dropping tube and water run in from time to time to prevent the volume in the flask from becoming less than 400 to 500 c.c.

"Distillate A, which contains the acetone preformed and aceto-acetic acid, and which should be collected in a second Kjeldahl flask, is redistilled (for about twenty minutes) after adding 10 c.c. of 10 per cent. sodium hydroxide†. The distillate so obtained (A_2) is titrated with standard iodine and thiosulphate solutions.

"The residue of urine plus sulphuric acid from which Distillate A was obtained is again distilled‡ dropping in either water, when necessary to keep the volume between 400 and 600 c.c., or a dilute solution of potassium bichromate. From 0.5 to 1 gram bichromate will usually be sufficient, and not more than 1 gram should be added unless the liquid turns green indicating a great reduc-

*If the urine contains but little or no sugar only half the amount or less of lead acetate should be used.

† In many instances, when a high degree of accuracy is not required, this redistillation may be omitted, and "distillate A" titrated direct; the results so obtained are slightly higher than those after redistillation from alkali.

‡ The distillation is actually not interrupted; after "A" has collected, a new receiving flask is adjusted and bichromate solution slowly added through the dropping tube. The receiving tube of the condenser must dip below the surface of the water in the receiving flask.

tion to chromium sulphate; very rarely 2 or 3 grams of bichromate may be necessary, especially if the sugar has not been completely removed.

"A 10 per cent. solution of potassium bichromate is kept on hand and 10 c.c. of this, diluted to 100 c.c., are measured out for each determination. 20 c.c. of the dilute solution (0.2 gram $K_2Cr_2O_7$) are first added slowly through the dropping tube and then 10 c.c. portions every fifteen or twenty minutes until the whole has been added. Should the liquid become markedly green the bichromate must be added at correspondingly shorter intervals and in amount sufficient to maintain a slight red-yellow color of the chromic acid, which may be detected even in the presence of the green. The distillation is continued with moderate boiling for from two to three hours. The distillate (B), which should be collected in a liter flask to avoid transference, is again distilled for about twenty minutes after adding 10 c.c. of 10 per cent. sodium hydroxide and 25 c.c. of 3 per cent. hydrogen peroxide. The flask must be heated cautiously until the peroxide has decomposed. This distillate (B_2) is titrated with the standard iodine and thiosulphate.

1 c.c. of $\frac{N}{10}$ iodine equals 0.968 mgm. acetone equals 1.736 mgm. oxybutyric acid, or

1 c.c. of $\frac{1.035N}{10}$ iodine (equals 13.13 mgm. I_2) equals 1 mgm. acetone equals 1.793 mgm. oxybutyric acid."

The results for oxybutyric acid obtained by this method are uniformly 5 to 10 per cent. too low, owing to the fact that in the oxidation with bichromate some of the acetoacetic acid undergoes acid decomposition with the formation of acetic acid instead of acetone, and a correction of 10 per cent. should therefore be made.

AMYLASE DETERMINATION IN URINE OR OTHER FLUIDS. WOHLGEMUTH.

Principle: A series of dilutions of the fluid to be tested is made and the same amount of starch solution is added to each. After warming the tubes to about 38 C. for half an hour the

action of the enzyme is checked by immersion in ice water and the dilution of the fluid still containing enough enzyme completely to digest the starch to the dextrin stage is determined by the addition of iodine solution to each tube.

Reagents: 0.1% starch solution*.
N/50 iodine solution, freshly prepared; made from a stock solution.

Technique: To each of ten test tubes in a rack add 1 c.c. of normal saline solution, except to the first, in which place 1 c.c. of the fluid to be tested. With a 1 c.c. pipette graduated in one-tenths add one c.c. of the fluid to be tested to the second tube, thoroughly mixing the two tubes. From this tube take 1 c.c. of the mixture and place in tube 3, continuing the process of mixing and withdrawing 1 c.c. from each tube in the series. The one c.c. withdrawn from the last tube is discarded. In this way the following series of dilutions is produced:

No. 1 = 1 c.c.	No. 6 = 0.032
No. 2 = 0.5	No. 7 = 0.016
No. 3 = 0.25	No. 8 = 0.08
No. 4 = 0.125	No. 9 = 0.004
No. 5 = 0.0625	No. 10 = 0.002

To each tube add two c.c. of 0.1 per cent. starch solution, mix, and place rack in the water bath at exactly 38 C. for exactly half an hour. The temperature must not vary more than one-half a degree above or below 38 C. The tubes are then removed from the water bath and placed at once in ice water to inhibit further action for five minutes. Two or more drops of N/50 iodine solution, as may be needed to bring out a permanent color, are added to each tube, taking care to use exactly the same number of drops in each case. The reading is made from the tube in which all the starch has been converted to the dextrin stage, that is, in which the iodine produces no shade of blue. To determine this, select the first tube in which any blue tone is appreciable and take the tube next preceding as the one showing the end reaction.

Result: The result is expressed in terms of the number of c.c. of 0.1% starch solution digested by 1 c.c. of the fluid to be tested

*Made from Kahlbaum's soluble starch. It should be freshly prepared every three days and kept in the refrigerator.

under the conditions of the determination. It is calculated as follows:

$$X = \frac{S}{U}$$

X = c.c. of 0.1 per cent. starch solution digested by one c.c. of the fluid tested.

U = c.c. of the fluid to be tested contained in tube showing end reaction.

S = c.c. of starch solution digested in tube showing end reaction.

Reports are made as follows:

$d\ \frac{38°}{30} = X$, or as $D\ \frac{38°}{24} = X$, according as digestion is allowed to go on for thirty minutes or twenty-four hours at the temperature stated.

Normal urine usually gives a value of about 16.

PHENOLSULPHONEPHTHALEIN TEST FOR RENAL FUNCTION. ROWNTREE AND GERAGHTY.

Principle: A measured amount of a dye which under normal conditions is very rapidly excreted by the kidneys is injected subcutaneously. The amount present in the urine voided at the end of two hours is determined by comparison in the colorimeter with a standard solution.

Reagents: Phenolsulphonephthalein solution in ampoules.
Standard solution of the same containing 0.006 gm. to the liter.
10% solution of sodium carbonate.

Technique: The patient drinks a glass of water and about half an hour later the bladder is emptied, either spontaneously or by catheter. Exactly 1 c.c. of the solution from an ampoule is then injected subcutaneously into some part of the trunk free from edema, preferably the lumbar region, using for the purpose an accurately graduated syringe. At the end of two hours from the time of injection the patient empties the bladder again or is catheterized and the urine is collected. In some cases a catheter is placed in position when the injection is given and the urine as

it trickles out is collected in a receptacle containing a few drops of 10% sodium carbonate solution. The time of the first development of a pink coloration of the fluid in the receptacle is noted, as this indicates the period elapsing before the first elimination of the dye. In acid urine the dye remains colorless, therefore the entire two hour amount of urine is first made strongly alkaline with sodium carbonate solution, causing it to become more or less intensely red according to the amount present. It is then diluted to a definite volume giving an intensity of color as nearly as possible like that of the standard and enough is filtered to fill the cup of the Hellige or Duboscq colorimeter. The former gives the percentage directly (which must be corrected for the dilution); with the latter the calculation is made as follows:

Result:

$$X = \frac{S}{R} \times \frac{Rd}{Sd} \times 100$$

X = percentage of phenolsulphonephthalein.
S = reading of standard.
R = reading of urine.
Rd = dilution of urine.
Sd = dilution of standard.

If the standard is set at 10 and the dilution of the standard is to 1000 the formula becomes $X = \frac{Rd}{R}$, that is, the percentage equals the dilution of the urine divided by the reading of the urine. In healthy persons the drug usually appears in the urine in from 5 to 10 minutes, and the total excretion at the end of two hours is from 50 to 85 per cent.

DIRECTIONS FOR COLLECTING BLOOD FOR ANALYSIS.

When the uric acid content of the blood is to be determined, the patient should be on a purin-free diet for at least two days before the sample is taken.

When the urea and non-protein nitrogen are to be determined, the patient should be on a low protein diet for at least two days before the sample is taken.

Blood samples are always to be taken before the patient has

breakfast. Blood in which the sugar is to be determined must be sent to the laboratory immediately after collection, as delay renders the results inaccurate.

The following amounts are necessary:

Urea and non-protein nitrogen	15 c.c.
Uric acid	20 c.c.
Sugar and creatinine	10 c.c.
All the above in one specimen	35 c.c.

A suction apparatus and graduated bottles for collecting the blood and a supply of powdered potassium oxalate will be furnished by the laboratory.

The rubber stopper with suction tubes attached is sterilized by boiling. According to the amount of blood to be drawn, place one or two knife points of the oxalate in the bottle, insert the stopper, and introduce the needle into the vein.

Aspirate the blood by suction on the mouth piece, shaking the bottle from time to time to prevent clotting. Stopper the bottle with a cork and shake *thoroughly*. Label as follows:

Name
Date
Examination required
Diagnosis
Ward
Hour taken

MCLEAN COEFFICIENT.

When this is to be determined, the following directions must be observed:

The patient is to be given 150 to 250 c.c. of water to insure a free flow of urine. One hour later the bladder is emptied, by catheter if necessary, and the time is noted to within one minute. About 36 minutes later 10 to 15 c.c. of blood is withdrawn and prevented from clotting by the use of potassium oxalate as usual. At the end of exactly 72 minutes from the time of voiding the bladder is again emptied and the entire specimen, taking care to avoid the least loss, is at once sent to the laboratory, together with the specimen of blood. The specimens must reach the laboratory before 10 A. M. The patient must take no food or drink during the 72 minute period. The patient's weight, taken on the day of the test, must be stated on the label of the blood specimen.

DETERMINATION OF TOTAL NON-PROTEIN NITROGEN IN BLOOD.

Folin and Denis.

Principle: The protein substances of the blood are precipitated by the action of an excess of methyl alcohol, complete clarification being secured by the addition of zinc chloride. The nitrogen in an aliquot part of the filtrate is converted into ammonia by digestion with concentrated sulphuric acid and the ammonia is then transferred to an acid solution by aeration. The amount of ammonia may be determined either by Nesslerization or by titration with N/100 alkali solution.

Reagents: Methyl alcohol (acetone free).
Saturated alcoholic solution of zinc chloride.
Potassium sulphate.
5% solution of copper sulphate.
Kerosene.
Saturated solution of sodium hydrate.
Caprylic alcohol.
Nessler's solution*.
Concentrated H_2SO_4.
N/100 H_2SO_4.
N/100 NaOH.
Alizarin.
Standard ammonium sulphate solution†.

Technique: Five c.c. of the blood obtained by puncture from a vein and kept from clotting by the use of a little finely powdered potassium oxalate (about 0.1 gm. for 20 c.c. of blood) is allowed

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*Nessler's solution is made as follows:

Mercuric iodide	200 gm.
Potassium iodide	100 gm.
Potassium hydroxide	400 gm.

Rub the red iodide to a smooth paste with water and transfer to a two liter flask. Grind the potassium iodide to a powder in the same mortar and add to the iodide in the flask using about 800 c.c. of water. Dissolve the potassium hydroxide in about one liter of water, cool thoroughly, and then add with constant shaking to the mixture in the flask. Make up to volume. The solution usually becomes perfectly clear. Place in incubator at 37 to 40 C. over night or until the yellowish-white precipitate which may settle out is thoroughly dissolved and only a small amount of dark brownish-red precipitate remains. The solution is then ready to be siphoned off and used.

†Standard ammonium sulphate solution. This solution, of which 5 c.c. contains 1 mgm. of nitrogen, is made by dissolving either 0.944 gm. of ammonium sulphate or 0.0764 gm. of ammonium chloride of highest purity in 1000 c.c. of distilled water.

to flow into a 50 c.c. volumetric flask half filled with acetone-free methyl alcohol. The flask is filled to the mark with methyl alcohol and is well shaken. At the end of two hours the fluid is filtered and the filtrate containing the non-protein nitrogenous constituents is further clarified by the addition of a few drops of saturated alcoholic solution of zinc chloride and re-filtration at the expiration of 5 to 10 minutes. The filtrate should now be quite colorless. Ten c.c. of this (equivalent to 1 c.c. of blood) is pipetted into a Jena glass test tube about 200 x 20 mm. and 0.5 grams of potassium sulphate, two drops of 5% copper sulphate solution, one c.c. of concentrated sulphuric acid and three drops of kerosene are added. The mixture is heated in a water bath until the alcohol has been driven off and then over a micro-burner until digestion is complete, as shown by the mixture becoming perfectly clear. It is boiled for two minutes longer to insure complete breaking down of the organic compounds, is allowed to cool, and is then carefully diluted with about six c.c. of water. The ammonia is removed from this fluid by aeration and the addition of alkali. For this purpose a receiving cylinder is arranged containing 15 c.c. of water, and two drops of 10% hydrochloric acid. The test tube containing the digestion mixture is placed in the other cylinder of the aeration apparatus and three c.c. of saturated sodium hydroxide solution is added. According to the strength of the air current aeration is complete in from fifteen minutes to half an hour. The ammonia in the acid solution may be determined either by Nesslerization or by titration.

A. Nesslerization. Into a 100 c.c. cylinder put 5 c.c. of standard solution and 50 c.c. of distilled water. Dilute 10 c.c. of Nessler's solution with 50 c.c. of water and add 25 c.c. of this mixture to the standard, then making up the volume to 100 c.c. with water. Without delay add 8 to 10 c.c. of the diluted Nessler's solution to the unknown solution in a 100 c.c. cylinder and at once dilute to 25, 50 or 100 c.c. according to the depth of color produced, using the dilution which comes nearest in depth to that of the standard. The standard solution is placed in one cup of the Duboscq colorimeter and the unknown is compared with it.

Result: $X = S \times \frac{Rd}{R}$

X = mg. of non-protein nitrogen per 100 c.c. of blood.

S = reading of standard.
R = reading of unknown.
Rd = volume in c.c. to which the unknown is diluted.

B. If the ammonia is to be determined by titration the receiving cylinder of the aeration apparatus must contain 20 c.c. of N/100 hydrochloric acid. The amount of this left un-neutralized by the ammonia formed in the blood solution is then determined by titration with N/100 sodium hydrate solution, using alizarin as indicator.

Result: Each c.c. of N/100 ammonia is equivalent to 0.00014 gm. of nitrogen.

The non-protein nitrogen in health is usually from 25 to 30 mgm. per 100 c.c. of blood.

DETERMINATION OF UREA IN BLOOD

VAN SLYKE AND CULLEN.

Principle: Urease, an enzyme obtained from the soy bean, converts urea into ammonium carbonate. It acts at room temperature, but is most active at a temperature of about 55 C. and when in a perfectly neutral solution. It decomposes nothing but urea, and is not interfered with by the presence of any other substance occurring in the blood. The urea in the blood is converted into ammonium carbonate by the enzyme, the ammonia in the latter is then liberated by the addition of alkali, and is aspirated into a measured amount of standard acid. The ammonium carbonate formed would by its alkaline reaction retard the action of the enzyme, but the development of alkalinity is prevented by the presence of potassium acid phosphate (KH_2PO_4). An excess of this, however, interferes with the combination of enzyme and urea which precedes hydrolysis of the latter so that it is advisable to use the minimum amount of phosphate that will keep the reaction sufficiently near the neutral point.

Reagents:
Soy bean enzyme (Urease)*
N/100 H_2SO_4
N/100 NaOH
0.6% solution of acid potassium phosphate, (KH_2PO_4)

*See determination of urea in urine.

Alizarin
Nessler's solution
Saturated solution of potassium carbonate. (90 grams to 100 c.c. water)
Standard solution of ammonium sulphate (1 mgm. of nitrogen to 5 c.c.)
Caprylic alcohol

Technique: Into a large test tube introduce 3 c.c. of 0.6% KH_2PO_4 solution, and 1 c.c. of 10% soy bean urease solution. Add 2 c.c. of blood drawn from a vein and prevented from coagulation by the addition of potassium oxalate (see non-protein nitrogen determination). Add 4 to 5 drops of caprylic alcohol to prevent foaming and allow to stand for 15 minutes. Then arrange the aeration apparatus as for non-protein nitrogen determination, add an equal volume of saturated potassium carbonate solution to the mixture and aerate for 20 minutes to one half hour. The nitrogen is determined by Nesslerization or by titration, as described under non-protein nitrogen.

Result: Nesslerization.

$$X = \frac{S}{R} \times \frac{Rd}{2}$$

X = mgm. of urea nitrogen per 100 c.c. of blood.
S = reading of standard.
R = reading of unknown.
Rd = dilution of unknown.

If the result is to be determined by titration place in the receiving cylinder 15 c.c. of N/100 acid and titrate back with N/100 alkali. Each c.c. of acid neutralized by the ammonia formed is equivalent to 0.00014 gm. N or 0.3 mg. of urea. The amount of urea may also be determined by multiplying the urea nitrogen by the factor 2.14.

Theoretically a second determination should be done without the use of urease in order to determine the ammonia alone in the blood so that this may be subtracted from the amount obtained from the tube containing the solution treated with urease. This fraction is however extremely small and ordinarily may be neglected.

The urea N of the blood in health is ordinarily about 11 to 15 mgm. per 100 c.c.

DETERMINATION OF URIC ACID IN BLOOD.

FOLIN AND DENIS.

Principle: The protein matter is removed from the blood and the uric acid is precipitated from the resulting clear solution by the addition of ammoniacal silver solution. This precipitate is re-dissolved with potassium cyanide and the resulting solution is treated with phosphotungstate reagent which gives a blue color proportional in depth to the amount of uric acid present. This is compared in the colorimeter with the color produced by a standard solution of uric acid under similar conditions (see determination of uric acid in urine.)

Reagents: N/100 acetic acid.
Alumina cream.*
Ammoniacal silver magnesia solution.
5% potassium cyanide solution.
Uric acid reagent.
20% sodium carbonate solution.
Uric acid standard solution.

Technique: To 100 c.c. of boiling N/100 acetic acid in a casserole add slowly 15 to 20 c.c. of blood carefully measured, and bring to the boiling point. Add 5 to 8 c.c. of alumina cream and continue boiling until the precipitate separates completely and the fluid when examined by letting it drop from a stirring rod is water clear. Filter through a hardened filter, wash the precipitate back into the casserole, wash it with boiling water, and filter again. The filtrates, which should be perfectly clear, are concentrated on a water bath to a low volume, are united, and then brought down to a final volume of about 2 c.c. If any protein precipitates, it should be removed by centrifuging and washing the sediment. The fluid and washings, which should not exceed 10 c.c., are transferred quantitatively to a centrifuge tube and treated with 20 drops of ammoniacal silver magnesia solution. Mix thoroughly, allow to stand a few minutes in a cold place (refrigerator) and centrifuge. Pour off the supernatant fluid completely, inverting the tube over a piece of filter paper and

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*Alumina cream is made as follows:
5% sodium bicarbonate solution.....100
4% aluminum acetate solution.....200
Allow the aluminum hydroxide to settle and decant three times with distilled water. Preserve in the form of a fairly thick magma.

removing ammonia vapor by introducing into the mouth of the tube a rubber tube connected with a suction pump. Add one or two drops of 5% potassium cyanide solution, stir well, and then add a few drops of water and stir again. Add one or two c.c. of uric acid reagent according to the bulk of the precipitate and either 5 or 10 c.c. of 20% sodium carbonate solution as the case may be. In a 100 c.c. cylinder place 5 c.c. of the uric acid standard solution, add the same number of drops of potassium cyanide solution as were used for the unknown, 2 c.c. of uric acid reagent and 10 c.c. of 20% sodium carbonate solution. At the end of about half a minute dilute to 100 c.c. Transfer the blue unknown solution quantitatively to a 50 c.c. cylinder and dilute to 25 or 50 c.c. according to its depth of color. Compare with the standard in the Duboscq colorimeter.

Result:

$$X = \frac{S}{R} \times \frac{V}{W}$$

X = mgm. of uric acid per 100 c.c. of blood.
S = reading of standard.
R = reading of unknown.
V = volume to which unknown was diluted.
W = volume of blood used in analysis.

The blood in health usually contains 1 to 2 mgm. of uric acid per 100 c.c.

DETERMINATION OF SUGAR IN BLOOD.

LEWIS AND BENEDICT.

Principle: The blood is freed from protein by the use of picric acid. The glucose in the resulting filtrate when heated in the presence of picric acid and alkali gives rise to a reddish-brown color proportional in intensity to the amount of glucose present. By comparison with a standard solution in the Duboscq colorimeter the amount of sugar is determined.

Reagents: Picric acid.
10% sodium carbonate solution.
Picramic acid solution.*

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*The picramic acid solution is made as follows:

Picramic acid	60 mg.
Sodium carbonate anhydrous	100 mg.
Distilled water to make	1000 c.c.

The solution should be standardized before use so that it gives the

Technique: Two c.c. of blood, which has been prevented from clotting by the use of potassium oxalate is measured into a test tube with an Ostwald pipette. The pipette is rinsed with water and the amount made up to 10 c.c. After shaking thoroughly in order to lake the corpuscles about 0.2 gm. of picric acid is added to precipitate the protein and saturate the solution with the acid. Allow the mixture to stand for several minutes and then centrifuge. Filter the clear supernatant liquid through a small paper into a test tube and measure three c.c. of the filtrate into a test tube provided with marks at 10, 15, and 20 c.c. Add one c.c. of 10% sodium carbonate solution and heat in a water bath for 15 minutes to cause the color produced by the alkali, picric acid, and glucose to reach its full intensity. Allow the solution to cool and with distilled water dilute to 10, 15, or 20 c.c. in order to produce a color as nearly as possible like that of the standard. The blood solution is compared with the standard at once to avoid color changes and the result is calculated as follows:

Result:

$$X = \frac{S}{R} \times \frac{m}{W} \times 100$$

X = mgm. of dextrose per 100 c.c. of blood.
S = reading of standard.
R = reading of unknown.
m = mgm. of dextrose to which the standard corresponds.
W = number of c.c. of blood used in the determination.

W and m are each equal to 0.6 and therefore cancel, so that

$$X = \frac{S}{R} \times 100.$$

In health, the blood sugar is usually between 75 and 120 mgm. per 100 c.c.

DETERMINATION OF CREATININE IN BLOOD.
MYERS AND FINE.

Principle: The proteins are precipitated by the use of picric acid. The creatinine present gives a brownish color on the addi-

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same color in the colorimeter as a solution containing 0.6 mgm. of glucose treated with picric acid and sodium carbonate as described above and made up to 10 c.c.

tion of alkali and the amount can be determined by comparison with a standard solution in the Duboscq colorimeter.

Reagents: Picric acid.
10% sodium hydroxide solution.
Standard creatinine solution.*

Technique: Six c.c. of blood prevented from clotting by the use of potassium oxalate is diluted to 30 c.c. with distilled water in a large centrifuge tube. After the corpuscles have been laked one half to one gram of picric acid is added and the mixture is stirred thoroughly. After complete precipitation of the protein substances has taken place the mixture is centrifuged and the supernatant fluid is filtered off. Ten c.c. of the filtrate (equivalent to 2 c.c. of blood) are measured into a large test tube and one-half c.c. of 10% sodium hydroxide is added. To 10 c.c. of the standard creatinine solution add the same amount of alkali and compare the two in the Duboscq colorimeter. Enough filtrate will have been obtained for a sugar determination on the same specimen.

Result:

$$X = \frac{S}{R} \times \frac{Rd}{Sd} \times \frac{100}{W} \times \frac{1}{2}$$

X = mgm. of creatinine per 100 c.c. of blood.
S = reading of standard.
R = reading of unknown.
Rd = dilution of unknown.
Sd = dilution of standard.
W = amount of blood in c.c. taken for determination.

The amount of creatinine present in health is usually from one to two mgm. per 100 c.c. of blood.

1 mgm. of creatinine is equivalent to 0.0371 mgm. of N.

DETERMINATION OF CREATINE IN BLOOD

Principle: On heating creatine to 120 C. in the presence of acid it is dehydrated, and its anhydride, creatinine, is formed.

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*This solution is made as follows:
Dissolve an accurately weighed amount of creatinine in N/10 HCl (about 1 mgm. to 1 c.c.) and dilute this solution with saturated picric acid solution so that the finished product contains exactly 5 mgm. of creatinine per 100 c.c.

Reagents: Picric acid.
10% sodium hydroxide solution.
Standard creatinine solution.

Technique: Place 5 c.c. of the filtrate obtained as for the creatinine determination in a test tube and heat in the autoclave at 15 to 20 pounds pressure (1000 to 1250 gm. per square cm.) for 20 minutes. Cool, dilute to 10 c.c. and then proceed as described for creatinine determination.

Result: From the amount of creatinine so obtained deduct that from the unheated blood. The difference will be the creatine content of the blood in terms of creatinine. To obtain the amount of creatine multiply this figure by the factor 1.16. The amount of creatine in health is usually 5 to 10 mg. per 100 c.c. of blood.

DETERMINATION OF CHLORIDES IN BLOOD.

MCLEAN AND VAN SLYKE.

Principle: The proteins are removed from the blood plasma by coagulation by heat in acid solution in the presence of an excess of magnesium sulphate. The fluid is clarified by the addition of animal charcoal and in the clear filtrate the chlorides are determined by titration with potassium iodide. For this purpose an excess of standard silver nitrate solution is added, the resulting precipitate of silver chloride is removed by filtration, and the amount of silver remaining uncombined is determined by titration with potassium iodide in the presence of nitrous acid and starch. As soon as the iodine has combined with all the silver, any additional amount added gives a blue color with the starch.

Reagents: 10% magnesium sulphate solution
Blood charcoal (Merck's Reagent; chloride free)
Silver nitrate solution (1 c.c. equivalent to 2 mgm. of NaCl.)

Silver nitrate	5.812 gm.
Nitric acid (sp. gr. 1.42)	250. c.c.
Water to	1000. c.c.

Potassium iodide solution (1 c.c. equivalent to 1 mgm. of NaCl.)

Potassium iodide	3.0 gm.
Water to	1000. c.c.

Starch solution

Sodium citrate ($Na_3C_6H_5O_7 + 5\frac{1}{2} H_2O$)	446.	gm.
Sodium nitrite	20.	gm.
Soluble starch	2.5	gm.
Water to	1000.	c.c.

Dissolve the starch in 500 c.c. of warm water, add the salts and heat till dissolved. While still hot filter through cotton, wash the filter with hot water, and after cooling, make the filtrate up to 1000 c.c. The solution becomes cloudy on standing, but keeps indefinitely.

The citrate and nitrite are present in order to secure the optimum conditions of acidity and to liberate the iodine from the iodide.

The potassium iodide solution is standardized against the silver solution by adding 5 c.c. of the starch solution to 5 c.c. of the silver solution and titrating with the iodide solution to the first appearance of a blue color. The iodide solution is then diluted so that 10 c.c. are exactly equivalent to 5 c.c. of the silver solution.

Technique: Centrifuge 5 c.c. or more of oxalated blood (see determination of non-protein nitrogen), and with an Ostwald pipette measure 2 c.c. of the plasma so obtained into a 20 c.c. stoppered volumetric flask containing 10 c.c. of 10 per cent. magnesium sulphate solution, rinsing the pipette twice by drawing up the solution. Add two drops of 50 per cent. acetic acid, add water to the 20 c.c. mark, mix, stopper lightly, and heat in the water or steam bath to 100 C. for ten minutes. Allow to cool and mix with about 0.3 gm. of blood charcoal (Merck's Reagent) in a beaker. Filter through a dry folded filter until a water clear filtrate is obtained. Measure 10 c.c. of the filtrate into a 25 c.c. volumetric flask, add 5 c.c. of the silver nitrate solution (equivalent to 10 mg. of NaCl), and make up to 25 c.c. Add two drops of caprylic alcohol, stopper the flask, and invert several times. Allow to stand five minutes, and filter through a dry folded filter, obtaining a clear colorless filtrate. To 20 c.c. of the filtrate add an amount of the starch solution equal to the amount of silver solution present in the volume of filtrate used, e.g., in the

present instance (5 c.c. of silver solution diluted to 25 c.c. and 20 c.c. of filtrate taken) 4 c.c. of silver solution is present and 4 c.c. of starch solution must be added. Then titrate with the potassium iodide solution to the appearance of the first definite blue color.

Result: Using 20 c.c. of filtrate from the silver chloride,

$$X = \frac{12.5\ (8 - Y)}{F}$$

X = gm. of NaCl per liter
Y = c.c. of KI solution used in titration
F = c.c. of blood filtrate taken

The chlorides in human plasma are normally from 5.62 to 6.25 gm. of sodium chloride per liter, according to the amount ingested.

FAT DETERMINATION IN FECES.

Principle: The soaps present in the stool, which are insoluble in ether, are split by the addition of acid alcohol into free fatty acids and neutral salts. The dried material is then extracted in the Soxhlet apparatus, the result giving the total of the ether soluble bodies in the stool and including small amounts of cholesterin, lecithin, and coprosterin, etc.

Reagents: 1% hydrochloric acid in alcohol.
Ether.
Anhydrous ether.

Technique: A small quantity of acid alcohol is added to the dried and pulverized fecal material and thoroughly mixed. The mass is evaporated to dryness on the water bath and about 5 gm. of the resulting powder is placed in a paper extraction capsule and extracted with ether in the Soxhlet apparatus for 24 to 72 hours. The extract is evaporated to dryness and the residue is dissolved in anhydrous ether and filtered into a weighing bottle, the filter being washed with a little fresh ether which is added to the filtrate. The entire filtrate is then evaporated to dryness and the weight determined.

Result: In adults the daily loss of fat in the stools may be put at 5 gm., or from 15 to 30% of the dried residue, but these figures are subject to great modification according to the diet.

DETERMINATION OF FAT IN MILK.

BABCOCK.

Principle: By the action of strong sulphuric acid the protein of the milk is destroyed and the fat may easily be centrifugalized from the hot fluid. A mixture of amyl alcohol and hydrochloric acid is added to facilitate the separation of the fat.

Reagents: Babcock sulphuric acid (Sp. Gr. 1.825)*
Amyl alcohol mixture.†

Technique: *Thoroughly* mix the sample of milk. With the special pipette measure 17.6 c.c. (the average volume of 18 gm.) into each of two Babcock bottles of equal weight and add a similar volume of the Babcock sulphuric acid. Mix carefully by shaking the bottle with a rotary motion. Centrifuge immediately for about four minutes and add two c.c. of the amyl alcohol mixture and enough of a hot freshly made mixture of equal parts of concentrated sulphuric acid and water to bring the fluid to the neck of the bottle. Centrifuge again for two minutes, and then again add enough of the hot sulphuric acid and water mixture to bring the level of the fluid nearly to the top of the graduation in the neck. Make the reading with dividers from the top of the upper meniscus to the bottom of the lower one. For breast milk use the special small tubes requiring only 5 c.c. of milk. The average composition of milk is as follows:

	Human	Cow's
Fat	4.00	3.50
Sugar	7.00	4.30
Protein	1.50	4.00
Ash	0.20	0.70
Water	87.30	87.50

Total solids in each case are about 12.50.

COLLOIDAL GOLD REACTION

Principle: If a cerebrospinal fluid is diluted in geometrical progression with a weak neutral electrolyte and five volumes of clinical colloidal gold are added to each successive solution, a

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*Concentrated sulphuric acid, 96%, Sp. gr. 1.84.....1800 c.c.
Distilled water ..... 189 c.c.
N. B. Add acid to water, not vice versa.
†Amyl alcohol.
Concentrated hydrochloric acid: equal parts.

series of precipitation colors may be obtained which, when plotted as abscissæ against the dilutions as ordinates, will give a curve whose contour is characteristic for certain of the infectious diseases of the central nervous system and meninges.

Reagents: Clinical colloidal gold.
0.4% solution of sodium chloride.

Technique:

THE PREPARATION OF CLINICAL COLLOIDAL GOLD. (O. I. LEE.)

Reagents: 1% gold chloride acid solution
2% potassium carbonate solution
1% oxalic acid solution
2.5% solution of formalin containing approximately 37% of formaldehyde (5 c.c. of formalin plus 200 c.c. of distilled water).

The solutions should be made up just before use from fresh distilled water. The gold chloride of Merck and Company in sealed ampoules is recommended. Any high grade will be found satisfactory for the other chemicals.*

To 1 liter of distilled water in a narrow neck glass-stoppered flask free from scratches and cleaned by treatment with warm aqua regia† and subsequent thorough rinsing, add 10 c.c. of the 1 per cent. gold chloride, 7 c.c. of the 2 per cent. potassium car-

* In order that the correct amount of formaldehyde should be used, it is necessary to determine the value of the formalin.

Evaluation of Formalin.
Modified Method of Ronijn.

Reagents: N/10 iodine solution (12.692 gm. of resublimed iodine dissolved in 150 c.c. of 10% potassium iodide and made up to 1 liter.)
2 N sodium hydroxide solution.
N/10 sodium thiosulphate (24.85 gm. of pure crystallized $Na_2S_2O_3$ $5H_2O$ made up to 1 liter)
HCl concentrated.

Ten c.c. of the formalin is made up to 1 liter with distilled water and 5 c.c. of this solution is mixed with 25 c.c. of the decinormal iodine. Two N sodium hydroxide is added, drop by drop, until the liquid becomes clear yellow. After ten minutes, 2 c.c. of concentrated hydrochloric acid are added and the free iodine titrated with decinormal sodium thiosulphate. Two atoms of iodine are equivalent to one molecule of formaldehyde. If T equals the titration, and S the specific gravity of the formalin, then

$$\text{Per cent. of } CH_2O \text{ equals } \frac{75 - 3\ T}{S}$$

† Seven volumes of concentrated hydrochloric acid to two volumes of concentrated nitric acid.

bonate, 1.75 c.c. of 1 per cent. oxalic acid, and 0.83 c.c. of 2.5% formalin (37% CH_2O), or equivalent amount of 8.07 mg. of CH_2O per liter of colloidal gold made. An Ostwald pipette is used for measuring the last reagent. Mix thoroughly, and heat over a Bunsen burner to between 80 and 85 C. Then reduce the flame enough to keep the temperature constant. The thermometer used must have had all the filler removed from the etched markings. The wire gauze supporting the flask should be covered with a sheet of asbestos cardboard to prevent local reduction on that portion of the flask directly over the flame.

The solution will slowly develop a series of colors substantially as given below, though some of the shades are much more transient than others. The associated temperatures are merely typical and were recorded for a development lasting about one hour. The color changes come gradually in good colloidal gold solutions.

Color	Temperature Degrees C
Faint blue green	65
Pale blue green	74
Pale bluish lavender	78
Bluish lavender	79
Pinkish lavender	80
Bluish pink	82
Pale pink	83
Pink	84
Deep pink	84
Violet red	84
Bluish red	83
Deep bluish red	83
Deep ruby red	83
Dark ruby red (violet red in test tube or pipette)	83
Rebound or color transition	
Orange red (orange pink in test tube or pipette)	83

When the solution reaches its maximum depth of color, a remarkable lightening and change of color occurs within the space of a few seconds, and *until this rebound and transition of color has taken place the solution will be worthless for clinical*

purposes. After this point has been passed, the solution is allowed to cool spontaneously to room temperature.

If a solution is heavily clouded, pour it out and examine the flask for spots and streaks of reduced gold transmitting green, blue or violet light. If noticeably present, treat with warm aqua regia for a few minutes, rinse thoroughly, and repeat the experiment. A clear colloidal gold solution leaves an unspotted container.

If the solutions still continue to be turbid and show a strong brownish fluorescence, a series of half a dozen liters should be made with amounts of 2.5 per cent. formalin, decreasing and increasing by 0.05 c.c. from that originally used and notice taken in which direction improvement occurs.

A slight brownish dispersion is no bar to the usefulness of the colloidal gold, but a six inch layer should be transparent, and 5 c.c. should be completely precipitated in one hour by 1.7 c.c. of 1 per cent. sodium chloride.

The correct color of the finished solution is matched almost exactly in a test tube comparison by a solution of 5 c.c. of 20 per cent. by weight of cobaltous nitrate ($Co(NO_3)_2.6\ H_2O$ plus 0.30 c.c. of 0.25 per cent. of potassium dichromate ($K_2Cr_2O_7$) solution, plus 10 c.c. of water. If the solution falls away in the direction of the blue from this standard, it should be rejected.

The solution should be stored in the dark in a container of Pyrex or Jena glass cleaned with aqua regia.

Technique of Test: Into the first of a series of eleven heavy glass lipless test-tubes, measuring 11/16 x 6 inches, 1.8 c.c. of the 0.4 per cent. NaCl is measured and into each of the remaining ten, 1 c.c. Of the spinal fluid to be tested (clear and free of erythrocytes, sediment, or moulds), 0.2 c.c. is then introduced into the first tube with a 1 c.c. Ostwald pipette graduated to the tip and provided with a wide margin between the 1 c.c. mark and the top. One c.c. of the mixture is placed into the second tube, 1 c.c. of this mixture into the third, and so on up to and including the tenth tube, from which, however, the 1 c.c. withdrawn is rejected, leaving the eleventh tube as a color control. The concentration of the spinal fluid thus ranges from 1:10 in the first tube to 1:5120 in the tenth. To each of the eleven tubes is then added 5 c.c. of the colloidal gold solution measured with a short pipette, and the contents mixed by a rotary motion.

At the end of half an hour the color of each of the ten tubes is matched against a diffuse white background, with the nearest shade selected from the following color scale, using the eleventh tube for comparison.

Color	Numerical Color Value
Colorless	7
Pale blue	6
Steel blue	5
Blue	4
Blue violet Violet blue	3
Violet (Blue red Red blue)	2
Violet red Red violet	1
Red	0

The corresponding numerical color values (abscissæ) are then plotted on coordinate paper against the dilutions (ordinates), multiplying the ordinate values by some convenient factor (5/3), to elongate the curve formed by connecting the points in a continuous line. This constitutes the preliminary curve.

At the end of twenty-four hours the color values are again taken, and the curve obtained designated as final.

Notes on Interpretation of Curve

A normal spinal fluid always gives a straight line (0 0 0 0 0 0 0 0 0 0), but a straight line curve does not necessarily indicate that the patient's spinal cord is normal; for instance, myelitis has given 0 0 0 0 0 0 0 0 0 0. General paresis (final) usually gives complete precipitation in the first five, six, or seven tubes, followed by gradations towards the red with the higher dilutions unchanged, as 7 7 7 7 7 6 3 2 1 0. Often, however, there may be a reversion of color causing a characteristic step-ladder or terraced curve (7 6 1 3 3 3 2 0 0 0).

Tabes and lues are very similar, but speaking generally, the luetic curve is inclined to be narrower, more to the left, and not so high as that of tabes. Thus, lues may be 2 2 3 2 2 2 1 1 0 0, and tabes 2 2 3 4 3 2 1 0 0 0.

In meningitis, reduction (peak of curve) is almost always to the right of the center, or on the extreme right.

Example: pneumococcus meningitis 0 1 1 1 1 1 3 4 4 3.

Except in general paresis, values exceeding 4 are not likely to be obtained, and the preliminary and final curve will be found coincident or roughly parellel. In paresis, however, the first half of the preliminary curve often lags progressively behind the first half of the final curve. Preliminary 2 3 4 5 4 2 1 0 0 0; final 7 7 7 7 7 2 1 0 0 0.

DETECTION OF MERCURY IN EXCRETIONS.

Principle: The organic combination in which the mercury exists is broken down by oxidation with nascent chlorine. By electrolytic double decomposition, the metal is then caused to become deposited on a piece of copper wire and from this it is distilled on to a piece of dentists' gold foil. The amalgam so formed is recognizable as a silvery patch of discoloration on the gold. In collecting the specimens and in carrying out the tests it is necessary to use only receptacles and apparatus that are chemically clean.

Reagents: Potassium chlorate
Concentrated HCl
Bare copper wire (B & S No. 14 is a convenient size)
Soft glass tubes, 2 mm. inside diameter, 10 cm. long, sealed at one end.
Small pellets of gold foil as used by dentists.*
Microburner.

Technique: As large a volume as possible of the material (urine, gastric lavage fluid, colon irrigation) is acidulated with 10 to 20 c.c. of concentrated hydrochloric acid; a few grams of potassium chlorate are added; and the whole is heated in a large porcelain evaporating dish. It is not advisable to use more acid or chlorate than is required for complete decolorization, the exact amounts being readily judged by experience. Stools, vomitus, blood, etc., are first diluted with several volumes of water, and require more of the oxidizing materials. The excess of acid and chlorine is eliminated by evaporation, and the solution concentrated to about 25 c.c. Any solid matter, especially fat, is filtered off; and into the filtrate, contained in an Erlenmeyer flask, is

*Manufactured by J. M. Ney Co., Hartford, Conn. Sold by dentists' supply firms in 1/16 oz. vials under the trade designation No. 1/4 velvet cohesive gold cylinders.

dropped a straight piece of the copper wire, 2 cm. long, previously cleaned by a short immersion in concentrated nitric acid followed by washing with distilled water. It is allowed to remain in the fluid for several hours or over night, preferably in a warm place. In an emergency the time may be shortened by boiling the fluid with the wire for five minutes as a preliminary test, but if the result is negative the test must be repeated in the regular way. The wire is washed with distilled water by decantation, dried by rolling very gently on a filter paper, and slipped to the bottom of the glass tube, avoiding abrasion. It is then followed by a cylinder of gold foil pushed to within 2 cm. of the wire.

Holding the tube horizontally, its closed end is carefully heated in the flame of the microburner almost to the softening point, after which the tube is gently warmed close to the gold cylinder, the edges of which should be frequently examined during this operation for any trace of a silvery discoloration signifying the presence of mercury. A hand lens is useful in recognizing very small amounts of the metal. If chlorine is still present in the concentrated oxidized solution the wire may be completely dissolved, in which case the solution should be diluted, again concentrated by boiling, and another wire dropped in. If the gold is too close to the wire, or the tube is heated too strongly, the mercury may be driven into or beyond the gold, the discolorization then being transient.

By this method it is possible to recognize the presence of 0.01 mg. of mercury in the amount of material taken for analysis. Calomel in therapeutic doses occasionally gives a positive reaction in the urine.

BACTERIOLOGY

GENERAL INSTRUCTIONS FOR LABORATORY WORKERS

The worker in the bacteriological laboratory of a general hospital is constantly handling infectious materials. Either through indifference or through lack of knowledge he may subject himself to serious consequences, as well as be the source of great danger to others. In order, therefore, to protect the worker and others against the possibility of infection, the following rules have been adopted and must be fully observed:

1. While in the laboratory, wear the regulation laboratory gown, which entirely covers the clothing. If infectious material is spilled on the gown, remove it and sterilize it in the autoclave.

2. Do not eat or drink in the bacteriological laboratory, nor place a pencil or other object in the mouth.

3. Moisten labels with water, not with the tongue.

4. Cleanse hands in disinfecting solution in basin, then wash thoroughly with soap and running water, before leaving the laboratory for meals or at end of work.

5. Do not leave infectious materials openly exposed.

6. Throw no waste materials on floor; place them in the pails or jars provided. Never throw infected materials into these receptacles.

7. Do not place stoppers removed from tubes or flasks containing live cultures on work table or other object, and always return stopper to tube or flask before discarding.

8. After finishing with tubes, flasks, or plates containing live cultures, place them in the small boiler which is provided at each work table. Keep the boiler covered when not in use.

9. Pour no infectious material into the sink or drain.

10. If infectious material is spilled on work table or floor flood with 5 per cent. carbolic acid, and allow to remain at least

one hour. Boil or place in the autoclave other objects accidentally infected.

11. If the hands become accidentally contaminated with infectious material or after such material has been handled, at once disinfect by washing for some time in a 1:1,000 solution of bichloride of mercury, which is found in basin placed on the work table, then scrub with soap and running water. Do not wipe hands on towel until they are thoroughly disinfected.

12. In handling infectious material use only pipettes plugged with cotton. After using, sterilize by boiling or by submerging completely in 2.5 per cent. cresol solution in the cylinder which is placed on each work table.

13. After taking blood cultures from patients with infectious disease, disinfect syringes and needles either by boiling or by submerging for some hours in 2.5 per cent. cresol solution. Place the other apparatus used in making cultures, such as agar tubes, etc., in small boiler to be sterilized.

14. Place animals inoculated with infectious material in proper isolation cages in the animal room. These cages must be cleaned and disinfected after use.

15. Take precautions with the body of an animal dead from inoculation, to prevent the spread of infectious material.

16. Nail animal to be examined post-mortem on wooden board; place in large enamel-ware tray; and wash the skin thoroughly with disinfecting solution.

17. Immediately after use, place in the instrument sterilizer all instruments used in the autopsy of an animal, and sterilize by boiling for 20 to 30 minutes.

18. Immediately after making an autopsy cover the body of the animal, without removing it from the board, with a thin layer of absorbent cotton thoroughly wet with disinfecting solution. Then remove to basement and place animal in covered metal can to be cremated. Sterilize the tray by flooding the bottom with 2.5 per cent. cresol solution and allowing it to stand for a few hours.

19. Report at once to the bacteriologist in charge all accidents, such as spilling of infectious materials, or any injury, such as cutting or pricking of fingers, burns, etc.

20. Permission must be obtained from the head of the department before any culture is taken out of the laboratory.

USE OF STERILIZERS.

Arnold Sterilizer. This is used in the sterilization of media containing substances (sugar, etc.) which are decomposed by heating at higher temperatures.

Always add water to the depth of 7 to 10 cm. (about 3 to 4 in.) to supply-pan before starting gas flame. Do not place tubes or flasks in the chamber until steam flows rapidly into it. Count time from moment when the materials are placed in the sterilizer. Heat fluid media in tubes 20 minutes in flowing steam, in large flasks 30 minutes; agar in tubes 25 minutes, in large flasks 30 to 45 minutes. When the time period is up, open door and at once remove the tubes or flasks. Repeat the heatings on three consecutive days to sterilize media.

Autoclave. If the ordinary upright laboratory autoclave with gas heater is used, always see that at least 10 cm. (about 4 in.) of water is in the bottom before starting the flame. After adding sufficient water, put in objects to be sterilized, then screw the lid down evenly all around and open the valve in the middle of the lid; then start flame. Leave valve open until steam flows rapidly from vent. Do not leave valve open long, as the autoclave will boil dry. Close valve and fasten clamps until no steam escapes from edge of lid. Allow pressure to rise until the gauge registers 15 lbs.; then lower flame, and maintain at this point for 30 minutes. Shut off gas flame, allow to stand until *pressure gauge returns to zero*, then open valve, and unscrew lid.

In case a more complicated sterilizer, like that for sterilization of surgical dressings, etc., is used, follow the directions which are furnished with the machine.

Dry Heat Sterilizer. The dry heat method of sterilization is used for glassware, glass syringes, needles, instruments, etc. Never use it for anything which is moist, only for dry objects. Since this apparatus is not under the control of an automatic regulator, the temperature must be regulated through the size of the gas flame, therefore, watch carefully while in use. Place in chamber the articles for sterilization; close the door; turn on and light the gas. Allow a full flame until temperature rises to 165 to 170 C., then lower the flame and maintain that temperature

for one hour. Higher temperatures will char cotton, and must, therefore, be guarded against. When the time limit is up, turn off the flame, and allow the temperature to fall below 100 C. before opening the chamber.

PREPARATION OF NUTRIENT MEDIA.

GENERAL DIRECTIONS

THE TITRATION AND ADJUSTMENT OF REACTION OF MEDIA.

I. Apparatus required: Two or three 100 c.c. porcelain casseroles or 100 c.c. Erlenmeyer flasks; two 50 c.c. burettes, graduated in 0.10 to 0.05 c.c., with suitable holder for operation; one 1 c.c. pipette, graduated in 0.10 c.c. or less; two or three 5 c.c. pipettes, graduated in 0.10 c.c.; one Bunsen burner; one iron tripod, height 7 inches, outside diameter 5 inches, with asbestos-filled wire gauze; glass stirring rods.

II. Reagents required: Indicator, 0.5 per cent. phenolphthalein dissolved in 50 per cent. alcohol; volumetric solutions, N/20 NaOH, N/1 NaOH, and N/20 HCl.

III. Procedure: First, see that all of the apparatus is thoroughly cleansed, and that the measuring burettes and pipettes are also dried before receiving the reagents, etc. Fill one of the burettes with N/20 NaOH solution and the other with N/20 HCl solution. Place 45 c.c. of distilled water in either a 100 c.c. white porcelain casserole or a 100 c.c. Erlenmeyer flask. To this add 5 c.c. of the medium, measuring most accurately with a 5 c.c. graduated pipette. In the case of a medium containing gelatin or agar, heat the distilled water before adding the sample. Add 1 c.c. of the phenolphthalein indicator; mix thoroughly with stirring rod; place container on wire gauze over Bunsen burner flame; and boil mixture for two minutes. Since most culture media require relatively more alkali at the boiling point than at lower temperatures to show the "end reaction", the titration should be carried out at that temperature as a constant. In general, most media are acid; therefore, only the N/20 NaOH solution is required; add this drop by drop from the burette, while constantly stirring or shaking the test sample. The "end reaction" is indicated by a faint but distinct pink color, which is uniform throughout the solution and permanent. Again heat

the sample to the boiling point for ten to fifteen seconds, and, if the color disappears, add immediately more of the N/20 NaOH solution to the same end reaction. Take this as the final reading for corrections of the reaction of the medium. It is desirable to titrate two or more samples from each lot of medium, and take the average in adjusting its reaction. The acidity or alkalinity of a medium is expressed in terms of that quantity of a normal (N/1) volumetric solution which would bring 100 c.c. to the neutral point, using phenolphthalein as an indicator. If acid, the plus sign (+) is placed before the number expressing this amount, if alkaline, the minus sign (—) is used instead. The desired reaction of the medium is adjusted by calculating the amount of acid or alkali required in terms of normal solution (N/1) for the total volume, and adding this amount directly to it. For example, if 2.3 c.c. of N/20 NaOH were required to neutralize a 5 c.c. sample of a freshly prepared broth, for 100 c.c. of this broth 2.3 c.c. of N/1 NaOH would be required (+ 2.3 per cent.). The reaction desired was + 1.0 per cent.; and as the difference between this and the reaction found (+ 2.3 per cent.) is equivalent to + 1.3 per cent., therefore, 1.3 c.c. of N/1 NaOH must be added to each 100 c.c. of the original broth for the correction.

After adding the required amount of alkali or acid to adjust the reaction, mix the medium thoroughly and place it in a steaming Arnold sterilizer for twenty to thirty minutes, if it is subsequently to be sterilized by the intermittent method in the Arnold sterilizer. If the subsequent sterilization is to be done in the autoclave, however, the preliminary heating also should be done in the autoclave at the same temperature and pressure, otherwise the final sterilization might cause a heavy precipitate to separate in the medium. The heating causes most of the precipitated phosphates to separate completely and they may be removed later by filtering. Correct the volume for evaporation, and again titrate samples. If the reaction is not within the required limits, make further corrections; mix; return to the steaming Arnold sterilizer for five to ten minutes, or boil for five minutes over flame; correct volume; then filter. The boiling or sterilizing of media usually causes an increase in acidity, especially in meat infusion broth, less so in meat extract broth; therefore, an amount of alkali slightly in excess of the estimated amount may be

added in adjusting the reaction. No definite rule can be given to cover this condition, since a number of factors enter which are subject to variation; the percentage excess must be determined for each particular method.

CLEARING OF MEDIA.

After the heating following the adjustment of reaction, most fluid media are sufficiently clarified by passage through double folded heavy filter paper. Gelatin and agar media are best filtered through layers of absorbent cotton. Make this filter by taking ordinary cheese cloth, wash thoroughly, fold to form two or three thicknesses about 12 x 12 inches in size; place on this a layer from roll of absorbent cotton about 10 x 10 inches; and fit the whole into an 8 inch funnel, packing down toward the outlet and to the sides. When this is in position, gently decant the hot medium into this filter without disturbing the arrangement. It may be necessary to pass the first portions of the medium through two or three times before the cotton filter becomes filled sufficiently to give a clear filtrate. In some cases this does not give the desired clearness, and it, therefore, becomes necessary to use another procedure. For this purpose, egg albumin is employed, usually the white of one or two eggs per liter. Whip up the egg white in a small amount of water and thoroughly mix with the medium, which has had its reaction adjusted and has been cooled to a temperature of 60 C. or less. Put the mixture into straight side enamel-ware vessels which may be placed either in the Arnold sterilizer or in the autoclave for the heating and coagulation of the albumin. The temperature should be brought up gradually to the point which will be used later in the sterilization of the medium, thus preventing further precipitation in the filtered medium. Use the autoclave for the coagulation of the egg white if the medium is to be sterilized by that method, and at the same pressure; otherwise use the Arnold sterilizer. Heat for thirty minutes, then remove from sterilizer; if the coagulum has formed at the surface, skim off; strain the hot medium through cheese cloth, folded to form four layers; press out the medium; correct volume; titrate; and adjust reaction if necessary. In such case heat at boiling point for five minutes before filtering. Finally, filter through a cotton filter prepared as outlined above.

In media prepared with meat infusion broth, instead of meat extract broth, the clarification may be carried out without the addition of egg white by extracting the meat in the first place at a temperature under 60 C.; then at a later stage bringing up to the boiling point and precipitating the coagulable proteins present. This method should be used when possible, and will be outlined later.

PREPARATION OF GLASSWARE FOR MEDIA.

It is essential that all test tubes, bottles, flasks, etc., which are to receive media should be thoroughly washed, freed from impurities, and well dried. Test tubes should be firmly stoppered with plugs of long fiber cotton (not absorbent), folded so that no stringy ends project into the tube. The plug should extend into the tube ¾ to 1 inch, depending on the size of the tube, and a free end should be left which will permit its ready removal. For large tubes, flasks, etc., it is well to cover the cotton plug with one or two layers of muslin or cheese cloth. All the glassware is sterilized in the dry heat oven for one hour at 170 C.

TUBING OF MEDIA.

Test tubes may be conveniently filled by connecting a discharging tube to the stem of a 6 inch funnel by means of a 3 inch soft rubber tube of suitable size which is closed by a stopcock. In filling the tubes care must be taken to prevent any of the medium from soiling the portion of the tube where the cotton plug might come in contact with it. The standard 6 x ⅝ inch test tube is used for most of the fluid media and for the liquefiable media which are to be slanted, 5 or 6 c.c. of the former or 8 to 10 c.c. of the latter being placed in each tube. The solid media, such as Loeffler's blood-serum medium, are put up in smaller tubes, which receive an amount sufficient to make a slant extending about one-half of the length of the tube; the same applies to the egg media.

Bottles (8 oz.) for blood culture work are filled about one-third full (75 c.c.).

STERILIZATION OF MEDIA.

In general, the intermittent method of sterilization causes less alteration of the constituents in media and is to be preferred when

time permits. All media containing sugars or other easily altered substances should be sterilized in this way. The Arnold sterilizer is used; it should be actively steaming when the media is placed in it, and time should be taken at that point.

Fluid media in test tubes, fermentation tubes, or small flasks should be sterilized for twenty minutes on each of three consecutive days; in the interval the media should be kept in a warm place. Liquefiable media, such as dextrose-agar in tubes, should be given a somewhat longer time period (not to exceed thirty minutes) on each of three consecutive days. When the media is in larger containers a proportionately longer time period should be allowed. When sterilization is carried out in the autoclave, thirty minutes at fifteen to eighteen pounds pressure are necessary if the medium is contained in test tubes or small vessels; one hour at the same pressure if larger containers are used.

SPECIAL DIRECTIONS

Immediately following this, methods for the preparation of the more commonly used culture media will be outlined. For those media not ordinarily used in routine work, the original sources of publication must be consulted.

Media may be grouped conveniently under three main divisions, based upon their physical character: liquid, liquefiable, and solid. This classification will be followed for convenience of reference.

I. LIQUID MEDIA

Litmus Milk. Separate the cream in fresh milk by placing the milk in a flask and heating it for fifteen minutes in a steaming Arnold sterilizer, and then putting it in the ice box for twelve to fifteen hours. After this, remove the lower portion from the surface cream layer by siphoning it into another flask. Fat-free milk can be procured from some milk dealers and is to be preferred when available. To the fat-free milk, add sufficient litmus solution to give a deep lavender color. Place about 10 c.c. in each tube, and sterilize in Arnold sterilizer for twenty minutes on each of three consecutive days. Milk should not be used if its reaction is more than + 1.5 per cent., unless neutralized with sodium hydroxide.

Dunham's Peptone Solution. Place 1000 c.c. of distilled or clear tap water in an enamelled stew-pan; add 10 gm. of peptone (American) and 5 gm. of sodium chloride; dissolve by boiling a few minutes; then filter through a double thickness of filter paper. The reaction should not exceed + 1.0 per cent. Sterilize either in the steaming Arnold sterilizer for twenty minutes on three consecutive days, or in the autoclave at fifteen pounds for thirty minutes.

Neutral Red Lactose Peptone. To a peptone solution prepared as outlined above, add 1 per cent. of lactose, and 1 per cent. of a saturated aqueous solution of neutral red. Dissolve sugar by heating; filter through filter paper, place in fermentation tubes, and sterilize for twenty minutes on each of three consecutive days, in Arnold sterilizer.

Nitrate Broth. To 1000 c.c. of water (ammonia free) add 1 gm. of peptone and 2 gm. of nitrite-free potassium nitrate. Heat until dissolved. Filter through double layer of filter paper; tube; and sterilize in Arnold sterilizer for twenty minutes on each of three consecutive days.

Plain Broth or Bouillon. Either meat extract or meat infusion may be used in the preparation of this medium. The use of the former simplifies the procedure and for ordinary purposes gives satisfactory results. Meat extract broth is prepared as follows: Place 1000 c.c. of water in enamel stew-pan; to this add 10 gm. of Liebig's meat extract, 10 gm. of peptone (American), and 5 gm. of sodium chloride, and dissolve by heat while stirring constantly. Measure volume, and add water for loss by evaporation. After mixing thoroughly, titrate, and adjust reaction to + 1.0 per cent. Place in a flask, and heat for twenty to thirty minutes in either the Arnold sterilizer or the autoclave, depending upon the method to be used for the final sterilization (see General Directions). After this, correct volume again, and take reaction; make corrections for reaction if necessary, and boil again for five minutes. Filter the solution through double layer of paper; tube; and sterilize in autoclave, or in Arnold sterilizer by the intermittent method. To prepare meat infusion broth select lean meat; remove all fat, tendons, and fascia; chop fine; add 500 gm. to 1000 c.c. of either distilled or clear tap water in suitable container. Place this in ice box and allow to macerate for eigh-

teen to twenty-four hours; or place it in a water bath and keep at a temperature of 55 to 60 C. for one hour, stirring occasionally. Following either of these procedures, strain through wet cheese cloth, express the juice from the meat, and bring the volume to 1000 c.c. Place this in an enamel vessel; bring temperature up to 50 C. over flame; add 10 gm. of peptone, and 5 gm. of sodium chloride; and stir until dissolved. Titrate samples and adjust the reaction to + 1.0 per cent. Proceed from this point as outlined for meat extract broth.

Sugar-free Broth. A meat infusion broth is prepared as outlined above up to the point immediately following the heating of the infusion in the Arnold sterilizer for twenty to thirty minutes. Then, instead of proceeding to readjust the reaction, filter the meat infusion through a layer of absorbent cotton and cheese cloth, press out, and bring to original volume. Cool the infusion to room temperature and inoculate each liter with a twenty-four hour broth culture of B. coli communis. Stopper the flask with cotton plug; place flask in incubator at 37 C. for about eighteen hours, then remove and put in autoclave for one hour at fifteen to eighteen pounds pressure. After the sterilization, restore volume; titrate samples; adjust reaction to neutral point; then heat in steaming Arnold sterilizer for twenty to thirty minutes. Filter through double thickness of filter paper; repeat until filtrate is clear. If the broth is not to be used at once as medium, place it in flasks, plug with cotton, and sterilize in the usual way by the intermittent method.

Carbohydrate Broth Fermentation Media. Sugar-free broth prepared as above is the base for the fermentation media. The substances most commonly used in this connection are the monohexoses: dextrose, galactose and levulose; the bi-hexoses: lactose, maltose, and saccharose; the polysaccharid: dextrin; and the polyhydric alcohol: mannite. These substances, which must be of the highest purity, are generally used in 1 per cent. concentration.

Place 500 c.c. of sugar-free neutral broth in clean flasks; add 5 gm. of the desired fermentable substance; and dissolve by heating gently if necessary. At once tube in sterile fermentation tubes, and immediately start sterilization in the Arnold sterilizer.

Heat for twenty minutes in flowing steam for three consecutive days.

Carbohydrate Serum-Water Fermentation Media. Ox blood serum is used in the preparation of these media. The blood is collected in covered sterile enamel-ware pails from the animal at the abattoir, care being exercised to exclude all unnecessary contamination. The freshly collected blood is allowed to clot before being moved.

Later, free the clot from the sides of the vessel with a sterile rod, place in ice box for twenty-four to thirty-six hours for separation of serum. Remove clear serum from clot by means of a large sterile pipette. If not clear, place in large centrifuge tubes, throw down cells, then remove the supernatant serum. Measure the serum and to this add two or three times its volume of distilled water—usually the proportion of one to three gives satisfactory results. Heat the mixture for fifteen minutes in the steaming Arnold sterilizer. Aqueous litmus solution may be added, if desired, until the fluid shows a deep transparent blue. Divide the finished serum into measured lots, and add to each the desired sugar in proportion of 1 gm. to 100 c.c. Dissolve, place in tubes, and sterilize in the Arnold sterilizer in the regular way.

Inulin Serum-Water. If the inulin is free from resistant spores, add it directly to the serum-water, prepared as outlined above, 1 gm. to each 100 c.c. But when resistant spores are present, sterilize the inulin first. Add 10 gm. of inulin to 750 c.c. of distilled water, place in autoclave and heat for fifteen minutes at fifteen pounds pressure. Remove and cool the sterile solution; then add 250 c.c. of clear ox blood serum and mix thoroughly. Add sufficient litmus solution to bring to a deep transparent blue color. Put about 10 c.c. in the ordinary culture tubes, and sterilize in the Arnold sterilizer for twenty minutes on three consecutive days.

Jackson's Lactose-Bile Medium. To 1000 c.c. of sterilized undiluted ox-gall (or 110 gm. of dry, fresh ox-gall dissolved in 1000 c.c. of water) add 10 gm. of peptone and 10 gm. of lactose. Dissolve by heating; filter through cotton until clear; divide in fermentation tubes and sterilize in the Arnold sterilizer in the usual way. In case large quantities of material, such as milk or

water, are used for inoculation, place 40 c.c. of the medium in large fermentation tubes and sterilize as above.

Dextrose Broth. This medium, when used only for cultural purposes, need not be made from fermented infusion broth. Either meat extract or meat infusion broth prepared in the usual way may be used. The reaction should be adjusted so that it is neutral to phenolphthalein, although for some purposes it may be + 0.2 to + 0.5 per cent. To 1000 c.c. adjusted broth add 10 gm. of dextrose; dissolve; heat twenty minutes in the steaming Arnold sterilizer; and filter through double layers of filter paper. Tube and sterilize in the Arnold sterilizer for twenty minutes on three consecutive days.

Calcium Carbonate Dextrose Broth. Prepare dextrose broth as above, using meat infusion rather than meat extract broth as the base. To this add 1 per cent. powdered calcium carbonate (previously sterilized in hot air oven) or place small pieces of marble in the tubes, and add the dextrose broth. Sterilize as with the dextrose broth.

Dextrose Ascitic Broth. Prepare dextrose broth as above; tube; and sterilize in the usual way. To each tube add, by means of a sterile pipette under aseptic precautions, clear sterile ascitic fluid in the proportion of one part of the ascitic fluid to two or three parts of the dextrose broth. Place the tubes in the incubator at 37 C. for twenty-four to thirty-six hours to test their sterility.

II. LIQUEFIABLE MEDIA

Nutrient Gelatin. Meat infusion, or meat extract broth may be used in the preparation of this medium. Take 1000 c.c. of meat infusion broth, add 10 gm. of peptone, 5 gm. of sodium chloride, and 120 gm. of best Gold Label gelatin, and dissolve the ingredients at a temperature under 60 C. Titrate samples; adjust reaction to a neutral point (phenolphthalein); place in flask; and heat in steaming Arnold sterilizer for thirty minutes. After heating, correct volume; titrate samples; and adjust the reaction to + 1.0 per cent. If additional alkali has been required, heat again at boiling point for five minutes, then filter through absorbent cotton, repeat until clear. Titrate the medium and note the reaction.

If meat extract is used, add to 1000 c.c. of water, 10 gm. of Liebig's extract, 10 gm. of peptone, 5 gm. of sodium chloride, and 120 gm. of Gold Label gelatin, and dissolve under 60 C. Titrate samples; adjust reaction to neutral point; then add the whites of one or two eggs beaten up in a small amount of water; shake up in flask; and heat in steaming Arnold sterilizer for thirty minutes. Correct volume; titrate samples; adjust reaction to + 1.0 per cent. and heat at boiling point for five minutes if additional alkali has been required. Filter the medium through absorbent cotton, repeating the process until clear. Nutrient gelatin should never be heated more than is absolutely necessary. Sterilize in Arnold sterilizer for thirty minutes on each of three consecutive days.

Plain Nutrient Agar. Meat infusion or extract of meat broth may be used in the preparation of this medium. When the meat infusion broth is desired, it is prepared by making the infusion double strength (500 gm. of meat to 500 c.c. of water). In such case add 10 gm. of peptone and 5 gm. of sodium chloride to 500 c.c. of this infusion, and dissolve at a temperature under 60 C. To 500 c.c. of water add 20 gm. of thread agar and dissolve, either by boiling over a free flame, or, better, by placing in autoclave and heating for thirty minutes under fifteen pounds pressure. Cool the agar solution to 60 C. or slightly under; combine with the infusion broth at same temperature; bring total volume to 1000 c.c.; mix thoroughly; titrate samples; and adjust reaction to neutral point. Place this in the autoclave, and heat for thirty minutes at fifteen pounds pressure. Titrate samples; adjust reaction to about + 0.7 per cent.; and boil five to ten minutes, after which filter the medium through absorbent cotton, repeating until clear. Sterilize the medium either in the autoclave for thirty minutes at fifteen pounds pressure, or in the Arnold sterilizer for twenty to thirty minutes for tubes (longer for larger containers) on three consecutive days. Meat infusion media become acid on heating, so that a titration should be done after final sterilization; the reaction should be near + 1.0 per cent. A meat extract base is not so prone to become acid on heating, therefore, when this is used, adjust to desired reaction directly.

The preparation of plain nutrient agar with meat extract instead of the meat infusion is much more easily carried out, and

answers very well in most cases. To 1000 c.c. of water add 10 gm. of Liebig's meat extract, 10 gm. of peptone, 5 gm. of sodium chloride, and 20 gm. of thread agar. Place this mixture in autoclave and heat for thirty minutes at fifteen pounds pressure to dissolve. Bring to original volume; titrate samples; adjust reaction to + 0.9 per cent.; cool to 60 C.; then add the whites of one or two eggs dissolved in a small amount of water; and mix thoroughly. Again heat in the autoclave as before, after which filter the hot agar medium through absorbent cotton until clear. Tube; and sterilize either in the autoclave or Arnold sterilizer in the usual way. The final reaction of the sterilized medium should be about + 1.0 per cent.

In some cases a "salt-free agar" is desired. This is prepared as above with the exception that the sodium chloride is omitted.

Dextrose Agar. Prepare plain nutrient agar as outlined above; adjust reactions from neutral to + 0.5 per cent., depending upon the particular use desired. To the clarified, filtered agar add 10 gm. of dextrose to each 1000 c.c. Tube in amounts for making slants and for plating; sterilize for twenty minutes on each of three consecutive days, in Arnold sterilizer.

Blood Agar. This medium is prepared by adding sterile, defibrinated blood to plain nutrient agar, either by mixing at a low temperature or by spreading over the surface of agar slants. The reaction of the agar for this purpose should be near the neutral point. Dissolve by heat the prepared agar medium and, by means of a pipette, measure 4.75 c.c. into each test tube. Sterilize in autoclave in the usual way. Cool the tubes to 50 C. in water bath, and to each add 0.25 c.c. of defibrinated blood by means of sterile pipette under aseptic conditions. Mix thoroughly, then slant to harden. After they are well hardened, place tubes in incubator (37 C.) for twenty-four to thirty-six hours to test sterility. If surface tubes are to be prepared, use plain agar slant tubes to which add one to two drops of defibrinated blood, spread well over the surface, and incubate to test sterility.

Rabbits' blood gives very satisfactory results in blood agar medium. To collect it bind a healthy animal on a suitable holder, and anesthetize it thoroughly with ether. Shave the neck over throat region, clean carefully, and sterilize skin as far as possible with 1:1000 solution of bichloride of mercury, or by painting with tincture of iodine. By means of sterile

instruments, make a longitudinal incision about 1.5 to 2 inches through the skin, and pull this aside to expose the muscles and trachea; divide the muscles by blunt dissection; and expose the carotid artery. Ligate the distal end of the artery, then clamp the proximal end and pass a ligature under to tie in position a glass cannula inserted in the artery through an opening made by means of fine tipped scissors midway between the two points indicated. When the sterile glass cannula is inserted and tied in the artery, remove the clamp, thus allowing the blood to flow out into sterile tubes or flasks containing coils of copper wire. Defibrinate the blood by shaking in these containers from ten to fifteen minutes. With care, the blood can be collected aseptically without difficulty, and can then be kept in a sterile condition in the ice box for future use.

Dextrose Ascitic Agar. Prepare dextrose agar as outlined above, and after final sterilization in the Arnold sterilizer cool in water bath to 60 C. By means of a sterile pipette, add sterile ascitic fluid in the proportion of one part of ascitic fluid to three parts of dextrose agar. Incubate at 37 C. for twenty-four to thirty-six hours to test sterility.

Starch Agar. Prepare beef infusion in the usual way, without the addition of peptone or salt. To 1000 c.c. of the infusion add 15 to 17.5 gm. of agar. Dissolve the agar by heating, correct volume, clarify, and filter. Titrate and adjust so that the final reaction will be 0.2 to 0.5 per cent. acid to phenolphthalein. Grind up 10 gm. of corn starch in a mortar with a little of the agar to avoid lumps; then add this to the main portion and boil for a few minutes. Tube in amounts for plating purposes, also for slants. Sterilize in autoclave for thirty minutes at a pressure not exceeding fifteen pounds.

Sodium Glycocholate Agar. To 1000 c.c. of water add 15 gm. of peptone and 15 gm. of agar, and dissolve by heating, either by boiling over flame, or in autoclave for thirty minutes at fifteen pounds pressure. Cool to 60 C., add 35 gm. of lactose and 5 gm. of sodium glycocholate; titrate; and adjust reaction to + 1.0 per cent. Then add the white of one or two eggs, (whipped up in a small amount of water); mix thoroughly; and place in steaming Arnold sterilizer for thirty minutes. Titrate again, after restoring to original volume, and correct reaction if necessary. In case more alkali must be added to readjust the reaction, heat again for five to ten minutes in the Arnold sterilizer, then filter through a layer of absorbent cotton, repeating until clear. Tube,

and sterilize, for twenty to thirty minutes on each of three consecutive days, in Arnold sterilizer.

Glycerin Agar. To 1000 c.c. of plain nutrient agar, prepared in the usual way, and adjusted to neutral reaction, add 50 c.c. of chemically pure glycerin. Tube, and sterilize in Arnold sterilizer for twenty to thirty minutes on each of three consecutive days.

Endo's Fuchsin Agar. To 1000 c.c. of water add 30 gm. of agar, 10 gm. of peptone, 10 gm. of Liebig's extract of beef, and 5 gm. of sodium chloride; dissolve by boiling or heating in the autoclave for thirty minutes at fifteen pounds pressure. Cool to 60 C.; add egg white to clear in the usual way, and filter to clearness through absorbent cotton. Correct volume, and make neutral to litmus (fresh papers) by the addition of sodium hydroxide; then add 10 c.c. of a 10 per cent. solution of sodium carbonate. Add 10 gm. of lactose, and 5 c.c. of saturated alcoholic solution of fuchsin, freshly filtered; mix thoroughly; add 10 per cent. sodium sulphite solution until the color just disappears on solidifying; usually about 25 c.c. is required Add about 15 c.c. of the medium to each tube, and sterilize in the Arnold sterilizer for twenty to thirty minutes by the regular intermittent method. Store in a dark place.

Kendall's modification of this method may be carried out as follows: Prepare 1000 c.c. of meat extract in usual way; make reaction slightly alkaline to litmus; divide in bottles (100 c.c. to each); sterilize, and store for stock. Mix medium and prepare fresh plates each time before using as follows: Add to each bottle 1 gm. of lactose; heat until dissolved, thus sterilizing the lactose; then add 1 c.c. of decolorized fuchsin, which has been prepared by adding 1 c.c. of saturated alcoholic solution of fuchsin to 10 c.c. of a 10 per cent. aqueous solution of sodium sulphite and heating in the Arnold sterilizer for twenty minutes. Pour the finished medium into plates and allow to harden. Place plates in the incubator to dry for thirty minutes, arranging them so as to protect them from dust.

North's Agar Gelatin Medium. Extract 500 gm. of lean chopped beef or veal with 500 c.c. of water by maceration for eighteen hours. Strain and press out juice; then add 10 gm. of agar, 20 gm. of Gold Label gelatin, 20 gm. of peptone, 5 gm. of

sodium chloride, and sufficient water to bring volume to 1000 c.c. Dissolve by heat and adjust the reaction to the neutral point for phenolphthalein. Heat again in the Arnold sterilizer, then filter through absorbent cotton until clear. Tube and sterilize in the Arnold sterilizer, by the intermittent method. If desired, 1 per cent. dextrose, which is more favorable for certain organisms, may be added.

III. SOLID MEDIA.

Plain Potato Medium. Select large potatoes and scrub them thoroughly with small brush under running water. By means of an apple corer, cut cylinders from the cleaned potato. Remove skin from each end of cylinder, and by an oblique cut divide into wedge-shaped portions with good butts. Drop them in clear running water, and wash for several hours. After this, drop a wedge, large end downward, into special potato tubes, or the ordinary straight tube, with a small wad of moist absorbent cotton in the bottom, sterilize in the Arnold sterilizer in flowing steam for twenty minutes on three consecutive days.

Glycerin Potato Medium. Prepare potato wedges as mentioned above, and place them in a 10 per cent. aqueous glycerin solution for several hours. Drop the wedges and a few drops of the glycerin solution into potato tubes and sterilize by placing in steaming Arnold sterilizer for twenty minutes on three consecutive days.

Loeffler's Blood-serum Medium. Separate clear serum from either clotted sheep or ox blood as outlined under the preparation of serum-water media. Prepare 1 per cent. dextrose broth from meat (beef or veal) infusion, and adjust reaction to neutral point with phenolphthalein as indicator. Mix three parts of the serum with one part of dextrose broth and place in small sized culture tubes a sufficient amount for slanting. Put these in a slanting position in a Koch serum coagulator and very gradually heat until a temperature of 90 C. is reached; hold the temperature at this point until the medium is completely solidified. In the absence of a Koch serum coagulator, the medium may be solidified either in a dry heat sterilizer, an autoclave, especially the horizontal type with steam jacket, or even an Arnold sterilizer. Success depends upon raising the temperature very slowly, and not

exceeding 90 to 95 C., thus preventing the formation of bubbles in the medium. After solidification of the medium, sterilize on the second and third days by placing in the Arnold sterilizer; slowly heat until the boiling point is reached, and hold there for twenty minutes.

Lubenau's Glycerin Egg Medium. Select ten perfectly fresh eggs, clean thoroughly with water, then wash with 5 per cent. carbolic acid. Allow to dry partially in the air; then dry both ends in the flame of a Bunsen burner. By means of a sterile (flamed) sharp pointed forceps make an opening of about 0.5 inch diameter through shell and membrane at one end; at the other end make a smaller opening through shell, if possible without breaking the membrane. Place lips to small opening and gently blow out contents of the egg into a sterile wide-mouthed flask or bottle. To the total contents of the ten eggs add 200 c.c. of neutral 5 per cent. glycerin broth. Mix thoroughly by gently shaking or stirring with sterile glass rod. Divide into test tubes by sterile plugged pipette; slant in serum coagulator; and heat at 70 C. for fully two hours on each of two consecutive days. If the medium is too dry after the sterilization, add two or three drops of sterile water to supply moisture. Incubate at 37 C. for twenty-four to thirty-six hours to test sterility.

PREPARATION OF STAINING FLUIDS, STAINING TECHNIQUE, ETC.

In general, the anilin dyes commonly used in the staining of bacteria are made up in concentrated or saturated solutions, either in distilled water or in 95 per cent. alcohol. Dilutions are made from such "stock solutions" after filtration for use in routine staining methods. The preparation of staining fluids may be conveniently considered under the following headings: (1) Concentrated Stock Solutions; (2) Simple Staining Solutions, Mordants, Etc.; (3) Special Stains and Staining Methods.

I. CONCENTRATED STOCK SOLUTIONS

Since considerable variation, both chemical and physical, exists between different samples of dyes appearing under the same name, no constant minimum can be given for the prepara-

tion of a saturated solution. The amounts given for the preparation of the following concentrated solutions are in part based upon the table prepared by Wood.

If a completely saturated solution is desired, the dye must be added until no more is soluble, as indicated by a slight excess of residue. The dyes used should be of the highest purity, especially prepared for bacteriological or medicinal purposes. Usually the dye is placed in the solvent at room temperature and dissolved by shaking thoroughly a number of times during several days. When desired for the preparation of the staining fluid, portions of the solution are removed and passed through soft filter paper.

Bismarck brown (Vesuvin), conc. aqueous solution. Add 0.5 gm. of Bismarck brown to each 100 c.c. of boiling distilled water. *Note*: A small piece of camphor may be added to preserve the solution.

Fuchsin (basic), conc. alcoholic solution. Add 3.0 gm. to each 100 c.c. of 95 per cent. alcohol, and dissolve.

Gentian violet, conc. alcoholic solution. Add 4.8 gm. to each 100 c.c. of 95 per cent. alcohol, and dissolve.

Methylene blue, conc. alcoholic solution. Add 7.0 gm. to each 100 c.c. of 95 per cent. alcohol, and dissolve.

II. SIMPLE STAINING SOLUTIONS, MORDANTS, ETC.

Acid alcohol. Add 2 gm. (volume 1.7 c.c.) of hydrochloric acid (sp. gr. 1.19) to 80 or 90 c.c. of 80 per cent. alcohol; mix; then bring volume up to 100 c.c. with the diluted alcohol. *Use*: Decolorizing agent. See stain for "acid-fast" bacilli.

Anilin-water. Add 5 c.c. of anilin oil to 125 c.c. of distilled water, thoroughly shake in flask, then filter through a double layer of filter paper well moistened with distilled water to retain undissolved anilin. *Use:* Preparation of anilin-water gentian violet. See Gram's stain.

Bismarck brown stain. Add 10 c.c. of filtered stock solution of the dye to 90 c.c. of distilled water. *Use:* As a counterstain apply one-half to one minute in Neisser or Gram methods.

Carbolic acid solution (5 per cent.). Add 5 gm. of chemically pure carbolic acid (phenol) to 95 c.c. of distilled water. Shake until dissolved. Filter. *Use:* Preparation of carbolfuchsin.

Copper sulphate solution. Dissolve 20 gm. of chemically pure

copper sulphate by shaking with about 75 c.c. of distilled water, then bring volume to 100 c.c. with water. *Use:* See Hiss capsule stain.

Fuchsin (basic), aqueous. Add 5 c.c. of filtered alcoholic stock solution of basic fuchsin to 95 c.c. of distilled water. *Use:* As a counterstain apply one-half to one minute.

Fuchsin, carbol. Add 10 c.c. of filtered alcoholic stock solution of basic fuchsin to 90 c.c. of 5 per cent. aqueous solution of chemically pure carbolic acid. *Use:* As a simple stain one-half to one minute. Also see spore stain and acid-fast bacilli stain.

Gentian violet, aqueous. Add 5 c.c. of filtered alcoholic stock solution of gentian violet to 95 c.c. of distilled water. *Use:* See Hiss capsule stain.

Gentian violet, anilin-water. Add 14 c.c. of saturated alcoholic solution of gentian violet to 126 c.c. freshly prepared anilin-water. Filter. *Note:* This stain should be freshly prepared every three to four days. *Use:* Principally in Gram's staining method.

Gram's iodine solution. Place 1 gm. of iodine and 2 gm. of potassium iodide in glass or porcelain mortar, and rub to a fine powder, gradually add a small amount of distilled water until all is dissolved, then bring total volume to 300 c.c. with distilled water. Filter. *Use:* Principally in Gram's staining method.

Potassium hydrate solution (1:10,000). Dissolve 1 gm. of potassium hydroxide in 100 c.c. of distilled water. Take 1 c.c. of the 1 per cent. solution just prepared and add 99 c.c. of distilled water to give the required dilution. *Use:* Preparation of Loeffler's methylene blue stain.

Sulphuric acid solution, (5 per cent.) Add slowly 5 gm. (volume, 2.7 c.c.) of sulphuric acid (sp. gr. 1.84) to 80 to 90 c.c. of distilled water, then as the solution cools bring total volume to 100 c.c. *Use:* Decolorizing agent. See Ziehl-Neelsen staining method.

III. SPECIAL STAINS AND STAINING METHODS

Capsule stain (Hiss). Smear material, such as sputum, chest fluid, etc., on clean cover slip or slide; if it contains no albuminous substance, place a drop of beef-blood serum on the slide, and spread. Dry in air and fix with heat. Cover with aqueous

gentian violet and heat over full flame until steam rises for a second. Wash off staining fluid with copper sulphate solution. Blot dry without washing in water, and examine.

Flagella stain (*Loeffler*). Use young growth from agar culture by gently mixing a small amount in a large drop of physiological salt solution on a perfectly clean, flamed cover slip. Dry quickly over flame. Fix in flame, but do not overheat. Prepare a mordant solution as follows:

Aqueous solution (20 per cent.) tannic acid,	10 c.c.
Saturated solution of ferrous sulphate,	5 c.c.
Saturated alcoholic solution of fuchsin,	1 c.c.

Filter at once after mixing, and use while fresh.

Pour mordant solution over fixed material and gently warm for one to five minutes. Wash in water. Stain with freshly filtered anilin-water gentian violet for one to two minutes; wash in water; dry; mount in balsam.

Gram's staining method. Make preparations of material or culture on cover slip (or slide); dry in air; fix in flame; and allow to cool. Cover with anilin-water gentian violet stain for five minutes at room temperature. Wash off staining fluid with Gram's iodine solution, allowing it to act for one minute. Wash off iodine solution with 95 per cent. alcohol, continuing this decolorizing process until the alcohol shows only a very faint violet tint. Wash in distilled water; counter-stain with either aqueous fuchsin or Bismarck brown solution for one minute; blot until dry.

Loeffler's alkaline methylene blue stain. Add 30 c.c. of saturated alcoholic solution of methylene blue to 100 c.c. of 1:10,000 aqueous solution of potassium hydrate. *Note:* Much better results are given if this stain is allowed to stand some weeks before using. *Use:* Apply stain for one minute or more to fixed preparation.

Neisser's acid methylene blue stain. Add 1 gm. of methylene blue to 20 c.c. of absolute alcohol, 50 c.c. of glacial acetic acid, and 1000 c.c. of distilled water; dissolve; and filter. *Use:* Apply to fixed preparation for two to five seconds, wash in water, and counterstain with Bismarck brown solution for five to ten seconds. Polar bodies appear blue, bacillary bodies brown.

Pappenheim's stain. In 100 c.c. of filtered saturated solution of methylene blue in absolute alcohol, dissolve 1 gm. of corallin (rosolic acid); then add 20 c.c. of glycerin. *Use:* This method is especially useful in the examination of materials for tubercle bacilli from sources where the smegma or other acid-fast bacilli may be present. Make smears of the material on slide and fix by heat in the usual way. Add carbolfuchsin solution and heat on hot plate or over flame until steam rises for two to three minutes. Pour off stain; then place in staining dish containing Pappenheim's stain; and allow to stand for one-half hour, occasionally shaking the dish. Remove, wash in water, and dry.

Spore stain. Make smears of old culture on cover slip or slide; dry, and fix with heat. Add carbolfuchsin; heat until staining fluid gently steams for three to five minutes; then wash in acid alcohol for about one minute. Wash in water; then counterstain with Loeffler's methylene blue for one-half minute; wash; dry. Spores appear red, while the body of the organism is blue.

Huntoon employs another method which is simpler, giving the double stain in a single step. Make a staining mixture as follows: Dissolve 4 gm. of acid fuchsin in 100 c.c. of 2 per cent. acetic acid; likewise dissolve 2 gm. of methylene blue (Grübler's) in 100 c.c. of 2 per cent. acetic acid. Mix the two solutions, filter to remove the resulting precipitate, and reserve the filtrate for staining. Cover the prepared, fixed smear with the staining solution, and steam over flame for one to two minutes. Wash in running water, dry and examine. Spores appear red, while the bodies of the bacteria stain blue.

Ziehl-Neelsen stain. Ziehl's carbolfuchsin is made as follows: In 100 c.c. of distilled water dissolve 1 gm. of basic fuchsin and 5 gm. of carbolic acid; filter the solution, then to the filtrate add 10 c.c. of absolute alcohol. Make thin smears of material for examination on cover slip or slide, dry and fix with heat; cover with the staining solution and heat until it gently steams for five minutes. Pour off stain, and decolorize by covering the stained preparation with 5 per cent. solution of sulphuric acid; immediately pour it off; repeat the procedure again; and wash in water, then in 90 per cent. alcohol, until no more color is dissolved. Others prefer to wash with acid alcohol until the preparation is practically decolorized. In either case, wash with water, then

counterstain with Loeffler's methylene blue solution for about one-half minute. Wash with water, dry and mount.

PREPARATION OF DISINFECTING SOLUTIONS

Carbolic acid, stock solution. Dissolve crystals of pure phenol by placing container, after the removal of stopper, in water-bath, and applying heat. To each 95 c.c. of the liquified phenol add 5 c.c. of glycerin, mix thoroughly, and place in glass-stoppered bottle. *Use:* Dilute for disinfecting solution.

Carbolic acid (C. P.) 5 per cent. solution. To 52.5 c.c. of stock solution of carbolic acid add sufficient water to bring total volume to 1000 c.c., and mix. Or weigh out 50 gm. of phenol crystals; add sufficient water to bring to 1000 c.c.; and dissolve.

Carbolic acid (crude) 3 per cent. solution. Dissolve about 25 gm. of commercial green soap in 400 to 500 c.c. of hot water, cool, then add 30 c.c. of crude carbolic acid; thoroughly mix; then add water to bring total volume to 1000 c.c.; mix.

Cresol, 2.5 per cent. solution. Add 25 c.c. of compound solution of cresol (U. S. P.) to 975 c.c. of hot water to dissolve.

Iodine, tincture (U. S. P.). Weigh out 70 gm. of iodine, 50 gm. of potassium iodide, place in mortar, rub up quickly into powder form, then place in glass-stoppered bottle. To this add in small amounts 95 per cent. alcohol, and shake until powder is dissolved. Finally add sufficient alcohol to bring the total volume to 1000 c.c.

Mercuric chloride, stock solution. Place 40 gm. of bichloride of mercury in a glass-stoppered bottle, add 1000 c.c. of distilled water, and shake until the salt is completely dissolved. *Use:* Dilute for disinfecting solution.

Mercuric chloride, 1:1,000 solution. To each 25 c.c. of stock solution of mercuric chloride add sufficient water to bring to 1000 c.c. Add concentrated methylene blue solution until the solution shows a light blue tint.

PREPARATION OF MISCELLANEOUS SOLUTIONS

Physiological salt solution, No. 1. Dissolve 8.5 gm. of chemically pure sodium chloride in 1000 c.c. of *freshly* distilled water, and filter through a double layer of filter paper. Place 125 c.c.

in each of eight 250 c.c. flasks, tightly stopper with cotton plugs covered with two layers of cheese cloth, cap with one or two layers of white paper. *Note:* Flasks must be thoroughly cleaned and then rinsed with two or three washings of freshly distilled water before the salt solution is placed in them. Sterilize the flasks with the contained salt solution in the autoclave at 15 lbs. pressure for one hour. When cool, label and store for use. *Use:* Diluent for therapeutic and diagnostic materials used in wards, for transfusion tests, etc.

Physiological salt solution, No. 2. Dissolve 9 gm. of chemically pure sodium chloride in 1000 c.c. of *freshly* distilled water in clean flask. Prepare just before using. *Use:* Washing of sheep corpuscles, preparation of corpuscle suspension, and in the dilution of active substance in connection with the Wassermann reaction. It need not be sterilized for this purpose.

Sodium citrate solution, No. 1. Dissolve 1 gm. of chemically pure sodium citrate and 0.85 gm. of chemically pure sodium chloride in 100 c.c. of distilled water. Filter; place in tubes; plug tightly with cotton; then sterilize in steaming Arnold sterilizer for twenty minutes on each of three consecutive days. *Use:* In collection of small amounts of blood for blood cells.

Sodium citrate solution, No. 2. Dissolve 10 gm. of chemically pure sodium citrate and 0.85 gm. of chemically pure sodium chloride in 100 c.c. of distilled water. Filter; tube; and sterilize as above. *Use:* To prevent coagulation of freshly drawn blood add one part of citrate solution to nine parts of whole blood. Mix well.

BACTERIOLOGICAL TECHNIQUE.

GENERAL DIRECTIONS

IMPORTANT: ALWAYS BURN OFF PLATINUM NEEDLE IN FLAME OF BUNSEN BURNER JUST BEFORE AND AFTER USING.

WATCH TEMPERATURE OF INCUBATOR CAREFULLY; KEEP IT AT 37 C. IF POSSIBLE, NEVER ALLOW IT TO APPROACH 40 C.; TOO LOW A TEMPERATURE IS LESS SERIOUS.

Cultures, materials for culture, and smears are generally brought to the Bacteriological Laboratory, but in some cases, when so desired, they may be taken by the bacteriologist in the ward.

The patient's name, the ward, the nature of specimen, and a note as to any special examination ordered should be entered in the bacteriological day book as soon as the specimen is received, for example: "Norrie III. Smith, John. Sputum for *B. influenzae*." It is well also to record the hospital case number to prevent confusion, for instance, when two patients with the same name are in the same ward, as has happened. Later, the diagnosis and findings are written in the book fully and clearly. The result of the examination is entered by the bacteriologist in the ward pathological record book under date of entry, with date on which culture was made, in parenthesis, giving the patient's name. A provisional report is made as soon as possible; thus, under January 2 "Smith, John. Culture from sputum (January 1) shows small Gram-negative bacilli." Under January 6, a final report is made: "Smith, John. Culture from sputum (January 1) shows *B. influenzae*."

Exudates, etc., should be inoculated on culture media as soon as received and at once placed in the incubator. Cultures in culture media also should be placed in incubator immediately on reaching the laboratory. Exudates not taken sterilly, when there is danger that the characteristic organism may be overgrown (for example, pus from supposed tuberculous sinus for inoculation into a guinea-pig), are placed in the icebox until the bacteriologist is ready to inoculate.

CARE OF LUER SYRINGE.

If the syringe has been used for infectious materials, disinfect, either by boiling or by immersing in 2.5 per cent. cresol solution, depending upon the nature of material. After disinfecting wash in water until clean. If water fails to remove blood, etc., use the sulphuric acid-potassium bichromate mixture, then wash thoroughly in running water. Dry; place plunger and barrel separately in large test tubes containing a wad of gauze in the bottom; plug tubes with cotton covered with two layers of gauze; place in dry heat oven; and sterilize at once. To prevent mistakes, never plug the tubes until just before placing them in the sterilizer. Sterilize for 1 hour at 170 to 180 C.

CARE OF HYPODERMIC NEEDLES.

After using with infectious materials (exudates, blood, etc.) wash needle while on syringe with 2.5 per cent. cresol solution by drawing in and discharging the solution several times; then boil in water. After using for injection of culture, disinfect needle at once by boiling. When disinfected, clean out the hub with a bit of cotton on forceps, and the lumen with fine wire. Sharpen points on soft stone, and polish with jeweler's emery cloth, or "Crocus" cloth. Dry lumen by passing first alcohol, then ether, through the needle. Place point downward in small test-tube containing a plug of gauze in the bottom. Do not plug the opening of the tube until just before sterilizing. Sterilize with dry heat at 170 to 180 C. for one hour.

ANIMAL INOCULATIONS.

Animal inoculations are made only when ordered. The procedure depends upon the nature of the material which is supplied. Solid materials and coagula from serous fluid generally are imbedded beneath the skin; sometimes they can be emulsified by rubbing them with sterile salt solution in a sterile tube, or they can be dissolved in 2 per cent. antiformin, and then injected. All fluids are injected into the animal by means of a sterile all-glass syringe with suitable needle. If there is a large quantity of fluid (urine, chest fluid, etc.) centrifuge it, remove the supernatant portion (disinfect), then take up the sediment in a syringe and inoculate the animal. Guinea-pigs are used for the recognition of tubercle bacilli; the inoculations are generally made

subcutaneously in the inguinal region. Mice are used to identify pneumococci by subcutaneous inoculation at root of tail.

Embedding Solid Materials Subcutaneously. Sterilize by boiling 3 or 4 straight forceps; 2 or 3 pairs of small scissors, needle forceps, curved surgical needles, etc. Place animal under ether anesthesia; shave the lower portion of the abdomen; then paint the dry skin with tincture of iodine. Grasp skin with sterile forceps forming a longitudinal fold over prepared area, with small sterile scissors cut through this fold to a depth of 0.75 to 1 cm., thus giving a transverse incision through the skin of 1.5 to 2 cm. in length. Thrust closed scissors (3 to 4 cm. blade) through opening downward between skin and muscular wall toward inguinal region about 5 cm. distance, then open blades, thus making a small subcutaneous pocket, close, and remove. With sterile forceps grasp edge of the skin at lower margin of the incision, raise from body wall, and by means of sterile forceps take up solid mass for inoculation and thrust under skin to bottom of pocket, release and remove forceps. Sew opening by approximating edges with forceps, and running close continuous stitch with cat-gut; handle needle with forceps to prevent infection. Clean the area with 5 per cent. carbolic acid.

Small openings may be closed by approximating edges of incision, drying the skin, painting over with collodion, covering with a very thin layer of absorbent cotton and over this placing another layer of collodion.

Sterilize all instruments by boiling immediately after using.

Inoculation by Injection. In general, fluids are injected subcutaneously into animals, but in some instances may be inoculated directly into the peritoneal cavity or into the blood stream. The hypodermic syringe used for inoculation should be sterilized by boiling for 15 to 20 minutes. For subcutaneous inoculation, the animal is prepared as outlined above. Take up the material in syringe through the needle; inject by puncturing the skin and passing the point of the needle toward the inguinal region, and discharge the contents between skin and body wall about 3 to 4 cm. from the point of entrance. Withdraw needle; disinfect puncture point with tincture of iodine; seal with collodion if necessary to prevent leakage.

In making an intraperitoneal injection, prepare the animal in the usual way; pinch up abdominal wall; and pass needle

obliquely through both skin and wall to avoid puncturing abdominal viscera. After injection, disinfect needle puncture. Injection of fluids directly into the circulation of animals is usually done by puncture of heart or into superficial veins. In the case of guinea-pigs, the heart method is most commonly used. Place the animal under ether anesthesia; remove the hair from chest by shaving; and disinfect skin with tincture of iodine. Pass the needle of syringe containing the material through chest wall just to right of sternum about third intercostal space, direct downward and backward about 3 to 4 cm. If the needle enters the heart, blood may be drawn into the syringe. Slowly eject contents from syringe. In larger animals, such as rabbits, shave hair from upper surface at outer margin of the ear; and disinfect with 5 per cent. carbolic acid. The marginal vein will be visible. Inject material in the usual way after passing needle of syringe through skin and into vessel, pointing toward the base of the ear. After removal of needle from vessel, place small wad of cotton over puncture point at once, holding the cotton in place with small artery forceps to prevent bleeding; remove later and sterilize by painting area with tincture of iodine.

Examination of Inoculated Animals. The attendant in charge of the animal room daily observes all the inoculated animals, and reports the results to the bacteriologist. All such animals which die are placed in a tray, and sent to the laboratory for examination. In general, to perform an autopsy on a small animal, such as a guinea-pig or mouse, proceed as follows: Sterilize by boiling 3 or 4 forceps, and the same number of scissors and scalpels. Also have an extra instrument sterilizer at hand to receive the infected instrument after using. Place the animal back downward on a board (about 15 x 25 cm.), distend legs and nail down by long tack through each foot. Do this in a large tray to prevent possible spreading of infectious material, and disinfect hands after handling the animal. Wash the skin of the animal with 5 per cent. carbolic acid, or 2.5 per cent. cresol solution. Lift skin in median line in pelvic region with forceps. With sterile scissors cut through skin only, and extend incision upward to neck. From each extremity of the median incision cut outward to each leg. Dissect skin from body with sterile scalpel, retracting with forceps. Examine site of injection (if subcutaneous), regional lymph-nodes, etc., and make cultures and

smears from subcutaneous exudate if present. Place instruments in reserve sterilizer. Make incision through abdominal wall in median line from pelvic region upward to diaphragm with sterile scissors. Make any necessary cultures or smears, and examine organs, etc. Open thorax with sterile heavy scissors by cutting through ribs from lower margin upwards on both sides laterally to the sternum; free the diaphragm; remove the wedge-shaped plate; then make cultures and smears (if necessary) and examine organs. To make cultures and smears from heart blood, place a sterile wire behind heart; tip forward; cut tip of ventricle with sterile scissors; then take blood with sterile Pasteur pipette or platinum loop; and proceed in usual way.

The routine procedure in a post-mortem examination depends much upon the particular type of organism under consideration. In the case of a guinea-pig inoculated with material suspected of containing the tubercle bacillus, the animal if it survives is usually killed five to six weeks after injection and examined. At autopsy examine for tubercles, especially at the site of injection and in the regional lymph-nodes, spleen, liver, lungs, and peritoneum. Make smears from tubercles found, stain, and examine for the bacillus. Take tissue from organs showing suspicious lesions, prepare stained sections and examine microscopically. Examine guinea-pigs dead from inoculation of material showing diphtheria-like organisms, particularly as to the character of the exudate at the site of injection, making smears and culture; examine also the peritoneal fluid; and the condition of the adrenals, noting whether they are hemorrhagic or not.

Animals inoculated with materials which are to be tested for other types of organisms are handled in similar manner at autopsy. Smears and cultures are made from exudates from various organs and from the heart blood and studied accordingly

SPECIAL DIRECTIONS

BLOOD CULTURES.

When a blood culture is ordered in the ward, make an appointment with the nurse in charge as to the time of taking it; also, if possible, get some knowledge of the suspected infection. In suspected typhoid infections, make half of the plate cultures with sodium glycocholate agar, and the remainder with plain agar; in suspected mycotic valvular lesions, etc., use dextrose

and plain agars for plating. In all cases inoculate a bottle of plain and one of dextrose broth with the blood.

Preparation of Patient. The nurse prepares the patient's arm for the bacteriologist just preceding the time of taking the blood for culture. Usually the following procedure is carried out: Select the arm which shows the most prominent veins at elbow. Clean the lower third of the patient's upper arm and the upper third of the forearm, with green soap, ether, alcohol, and a 1:1000 solution of bichloride of mercury. Apply the alcohol and bichloride of mercury solution with sterile gauze. Cover the area selected for vein puncture with a piece of sterile gauze moistened with the bichloride of mercury solution, until ready to draw blood for culturing. Do not touch this prepared area with the fingers until the needle is through the skin. If it becomes necessary to palpate the vein, do so through a piece of sterile gauze.

Apparatus and Materials Required. Assemble in tray a sterile 20 c.c. Luer syringe, several sterile syringe needles which must be clean and sharp, six sterile Petri dishes in carrier, ethyl chloride, alcohol lamp and matches, rubber tubing for tourniquet, and clamp. Place six agar tubes with thermometer in large enamel cup, add water and boil until agar is liquified, cool down to 55 C. before leaving for ward. Also include a plain and a dextrose broth flask, and a cup containing 2.5 per cent. cresol solution for disinfection of used syringe and needle.

Procedure. The nurse assists the interne in making the cultures by seeing that a small table covered with a sterile sheet is placed at bedside, arranging plates on table, passing instruments and syringe, and bringing the agar to a temperature of 40 to 42 C. After instruments, etc., are arranged, place tourniquet about upper arm to constrict vein, put plunger in barrel of syringe and securely fasten on the needle, taking care not to infect any of the essential parts. Remove the moistened gauze from the arm, insert needle into vein and draw about 10 c.c. of blood. Release tourniquet after syringe is filled before the needle is withdrawn. Usually the syringe containing the blood is passed to the assistant who adds it as directed to the medium while the interne does the mixing, pouring of plate cultures, etc. Take care not to force the needle off the syringe in ejecting the blood; also work rapidly to make all cultures before the blood coagulates. Add about 1 c.c. of blood to each agar tube, and after adding the blood, mix,

carefully flame the mouth of tube, and pour contents into Petri dish. To pour plates, raise cover of dish at one side just sufficient to admit the end of the tube without touching. Replace cover and spread fluid by slightly tipping and rotating the Petri dish. The remainder of the blood in the syringe is divided between the two broth flasks. After the blood is drawn the puncture in the patient's arm is cleaned, and covered with sterile gauze, which is fixed with adhesive straps.

In the majority of cases blood can be obtained by direct puncture of the vein through the skin. If this fails, a small incision may be made (under ethyl chloride anesthesia) obliquely to the course of the vein, which is then exposed to blunt dissection. This procedure is carried out aseptically, as any minor surgical operation. After the blood is withdrawn from the exposed vein, the incision is closed and surgically dressed, and care is taken to prevent any infection of the patient. After taking cultures, cleanse and disinfect syringe as previously outlined (page 112) and place test tubes in desk boiler for sterilization in autoclave later.

Care of Cultures, Etc. After the agar cultures have hardened, fasten the cover of the dish to the lower portion by wrapping a narrow strip of adhesive tape about the two. Write patient's name, ward, and date on each, and label each flask in the same way. Invert the plates, stack, and place in incubator along with flasks. Make record of case in the bacteriological record book, and also in special blood book where the daily records are entered. If nothing develops in the interval, blood cultures are observed for ten days. Examine cultures daily. If colonies develop on plates make transfers to broth tubes (usually dextrose), and make smears for Gram stain if there is sufficient material. If the broth flasks are turbid make smears for Gram stain, and make transplants to suitable media for type of organism present. If mixed cultures are found, plate material and isolate types. Report findings daily to ward whether positive or negative for first three days, and give final diagnosis as soon as made.

Anaerobic Blood Cultures. Occasionally anaerobic blood cultures are ordered. The procedure is carried out as outlined above, excepting in the preparation of plates and the making of anaerobic agar slant inoculations. Draw blood in the usual way; add about 1 c.c. to either plain or dextrose agar at 40 to 42 C.; and

pour in special deep dishes as used in the Zinsser method of making anaerobic plates. Or, prepare six Petri dish plates as outlined above; later place them in a Novy anaerobic jar; seal; displace air with hydrogen from a generator; then place in incubator for usual period of incubation. Also add about 1 c.c. of blood to each of several plain or dextrose agar slant tubes. The top of the slant should reach only about one-half way to the top of the tube. Spread well over surface, and either place in the Novy anaerobic jar or absorb the oxygen by the alkali-pyrogallic acid method. To carry out the latter simply cut off the cotton plug level with top of the tube, push it down almost to the upper end of the slanted medium, fill and pack the open portion of the tube with dry pyrogallic acid, add a few drops of concentrated solution of sodium hydroxide just sufficient to moisten the powder, then place a fresh cotton plug in the tube, cut top off, force downward, and seal at once with melted paraffin.

To prepare anaerobic plates by the Zinsser method, after the agar medium has hardened, invert the dish, lift out the bottom portion (containing agar culture) and place top downward on a filter paper wet with 1:1000 bichloride of mercury solution. This prevents air contamination. To the top portion of the dish, add pyrogallic acid to the depth of about 2 cm., then add 10 per cent. sodium hydroxide solution to fill dish. At once, partially place the bottom part, open portion downward, in this solution, allowing the contained air to escape in so far as possible. Level, and run liquid paraffin into the space between the walls of the two parts of the dish to seal from air.

If colonies develop, fish, and transplant to agar slants, culture under both aerobic and anaerobic conditions.

COLLECTION OF BLOOD AND SEPARATION OF SERUM FOR WASSERMANN TEST.

Expose patient's arm and place rubber tourniquet about upper portion, taking care not to shut off the pulse in the wrist entirely. Clean arm with green soap, ether, and alcohol, using sterile gauze to apply the alcohol, or disinfect the skin over the vein selected by painting surface with tincture of iodine, which seems to give equally good results. Do not contaminate the prepared area before the blood is withdrawn. Take a large needle (No. 19 gauge 1.5 in. long) which has been sterilized by dry heat

in plugged test tube and place on a dry all-glass syringe. Avoid touching the needle for at least one inch from the point. Insert the needle into the most prominent vein and draw about 10 c.c. of blood. If desired, the skin may be frozen with ethyl chloride before puncturing the vein. After drawing the blood, loosen tourniquet, withdraw needle, and cover the puncture at once with several layers of sterile gauze which is pressed down to stop flow of blood. Discharge blood from the syringe immediately into a sterile test-tube (about 12 cm. x 2.5 cm.); replace plug; place tube in slanting position; and allow blood to clot at room temperature. Clean syringe and needle before blood clots. Remove gauze from patient's arm, clean the arm with alcohol on sterile cotton, touch needle puncture with drop of tincture of iodine on small swab. Cover the area with several layers of sterile gauze, fix in position by means of adhesive strips, and instruct the patient to remove this on the following morning. Place a small (2 cm. x 5 cm.) adhesive strip on blood tube, and label plainly with patient's name, ward, and date. Enter these data in the bacteriological record book, also in the special Wassermann test book and give serial number. After clot is well formed, separate from walls of tube by means of sterile wire, then place in ice box. Remove serum from clot at end of day, place in a 15 c.c. conical centrifuge tube, and throw down all blood cells. See that this tube is properly labelled to prevent confusion or errors. After cells are thrown down, withdraw the clear serum; place in test-tube; label with serial number; stopper with cork; and store in ice box until tests are made. If possible, do not draw blood for Wassermann tests until the day preceeding the day of testing; fresh serum is desirable.

EAR CULTURES.

Make cultures on Loeffler's blood serum medium and dextrose broth. Also make two smears from discharge on clean glass slides. Stain one smear by the Gram method. If Gram-negative bacilli are present, stain the other smear for capsules by the Hiss method. Examine cultures on the following day in the usual way. If Gram-positive, medium sized, palisading bacilli are present, stain with Loeffler's methylene blue and Neisser's stain. To determine species, plate on plain agar, isolate in pure cultures, and prove types.

EYE CULTURES.

Make culture on Loeffler's medium and 5 per cent. blood agar and, if discharge is scant, on dextrose broth. Also make smears on clean slides, if discharge is sufficient, and stain by Gram method. Examine cultures on the following day and make Gram stain. If blood agar culture shows colonies characteristic of the hemoglobinophilic type of organism, use aqueous fuchsin as counterstain in the Gram method. For identification of ordinary types of organism, plate to isolate, fish colonies, and transplant to dextrose broth. Stain by Gram method, and prove types by cultural methods, etc.

Surface seedings on rabbit-blood agar plates are necessary for isolation of the hemoglobinophilic group. Fish fine, dewdrop-like colonies, and transplant to blood agar slant tubes.

THROAT AND NOSE CULTURES.

If diphtheria is suspected, make cultures from membrane on Loeffler blood serum medium, using sterile cotton on wire or wood rod. Also make smears on clean slides, air dry, fix in flame, and stain. Make both Loeffler methylene-blue and Neisser stains on smears and on cultures, then examine for diphtheria bacilli. Allow cultures to incubate from twelve to twenty-four hours, since *B. diphtheriae* seldom develops growth early. To prepare stains from cultures on Loeffler medium with sterile platinum needle remove growth (especially small, gray-white, rather dry-appearing colonies), and emulsify in a drop of sterile salt solution on clean slides. Spread emulsion until rather thin film is formed; air dry; fix in free flame; and stain as directed (p. 107), In doubtful cases, make a Gram stain which will differentiate certain small bacilli from true diphtheria bacilli, and also bring out streptococci more clearly.

Throat and nose cultures are taken from each child before admission to the hospital, and the growth is examined on the following morning. In case there is a visible lesion in the throat at the time the culture is taken, smears should be made on slides and examined for diphtheria bacilli, streptococci, fusiform bacilli, and spirilla. A diagnosis of diphtheria may sometimes be made by direct examination of the exudate, but one should not make a negative report without examining a culture. In Vincent's

angina, smears stained about 30 seconds with aqueous fuchsin usually show the fusiform bacilli and spirilla clearly.

PUS AND EXUDATE CULTURES.

Make smears on clean glass slides, stain one by Gram method, and another by Hiss capsule stain, if indicated by a study of the former. Make cultures on dextrose broth and Loeffler's medium. Centrifuge spinal fluid, clear chest fluid, etc., in a sterile tube and make cultures from the sediment. If either gonococcus or meningococcus is suspected, make cultures on dextrose ascitic agar and dextrose ascitic broth; if *B. influenzae* is suspected, make culture on 5 per cent. blood agar slants. It is often advantageous to incubate the fluid itself. Occasionally a small fibrin clot will separate; in such cases, inoculate medium with this portion. Isolate organisms in pure culture, and prove types.

URINE CULTURES.

Specimens for culture should be taken by catheterization and sent to the laboratory in sterile tubes, utmost care being taken to clean parts before drawing the specimen. In men, after the parts have been well cleaned, the last portion of urine voided may be collected in a sterile tube or directly into sterile broth.

Transfer 1 or 2 c.c. of urine to a dextrose broth tube and to a plain agar slant with a sterile Pasteur pipette. Centrifuge the remainder of the specimen, make smear and stain by Gram method. Examine cultures on the following day.

For identification of species in a growth, plate on plain agar in the usual way, fish different types of colonies, and inoculate into dextrose broth tubes. Make Gram stains of the isolated organisms; and be guided by findings for further cultural tests, etc.

STOOL CULTURES.

To examine stools for typhoid or dysentery bacilli, make a very dilute emulsion in broth. Add a drop of the emulsion to the first of three or four poured plates containing well hardened Endo's medium. By means of a sterile spatula or glass rod with end bent at right angle, spread the emulsion by streaking over each plate consecutively. Or, plate in the usual way on Endo's medium, pouring into large Petri dishes.

After twenty-four to forty-eight hours incubation both the typhoid and the dysentery bacillus appear as small translucent white colonies. Organisms of the colon group form red or pink colonies. Certain other organisms which do not produce red colonies may be present.

Make six to ten fishings from characteristic appearing colonies; inoculate on plain broth tubes; and incubate over night. Make Gram stains, and, if Gram-negative bacilli are found, examine in hanging drop for motility. Inoculate various sugar media for fermentation characteristics; also inoculate Dunham peptone for indol production, and litmus milk for reactions.

VAGINAL SMEARS.

A vaginal smear is made in the examining room from every girl before admission. The smears must be taken and examined immediately. To make smear, separate labia, introduce sterile swab into vagina, and smear by rolling motion on clean slide. Fix by passing three times through flame; stain by the Gram method; and examine. In each case the exact findings should be reported in the bacteriological record book; for example: "Mary X. Vaginal smear: Shows numerous rather degenerated pus cells, many Gram-positive cocci, also small Gram-negative bacilli; no Gram-negative intracellular diplococci found." Never report a case positive for gonococcus, but state that smear shows pus cells containing many Gram-negative intracellular diplococci. As a general rule, a few intracellular Gram-negative diplococci resembling the gonococcus are sufficient to exclude a child, even if not to make a definite diagnosis of gonorrhoea.

Smears that show leucocytes filled with morphologically typical Gram-negative diplococci may be considered positive. Leucocytes showing only an occasional intracellular Gram-negative diplococcus must never be called positive, but should be considered suspicious. If numerous pus cells are present, but no Gram-negative intracellular diplococci are found, the case should be subjected to further examination before giving a report.

EXAMINATION OF SPUTUM.

Examine these as ordered on accompanying tag; open the sputum cups at designated place under hood, and make required smears and cultures. Make stains for tubercle bacilli at same

place; make stains for predominating types of organisms, etc. at work desk; but fix smear on slide in flame before removing from hood. Do not ordinarily take sputum cups to any other part of the laboratory, but if this becomes necessary place them in a basin which can be disinfected. After examination run strong carbolic acid solution in cups, then place them in a pail containing the same solution. Precautions must be taken not to spill sputa, but if this accidentally occurs flood spots at once with 5 per cent. carbolic solution.

For B. influenzae. Make rather thin smears on clean slides, air dry, fix by heat, and stain by Gram method, but counterstain with aqueous fuchsin solution instead of Bismarck brown.

To cultivate the influenza bacillus arrange a series of five or six sterile Petri dishes; almost fill each with sterile physiological salt solution, then select from the sputum one or more of the denser masses by means of a sterile platinum loop, and transfer to the first of the series of dishes. Wash by stirring about with the loop, transfer to the second dish, and continue washing through the series of dishes until the outer portions of the small mass are removed. Place the washed particle on a sterile blood-agar plate, and with a sterile platinum or nichrome seeding spatula make consecutive streaks in parallel lines over the plate, finally continuing this procedure on a second blood-agar plate. If blood-agar plates are not available, the seeding may be carried out on a series of blood-agar tubes by means of a platinum loop. The influenza colony usually appears in about forty-eight hours as a minute, slightly raised, "dew drop" colony. Make fishings of characteristic colonies to blood-agar slants and incubate. If Gram stains made from cultures show the characteristic Gram-negative bacillus, make transplants to both blood-agar and hemoglobin-free-agar slants. If no development takes place within seventy-two hours on the hemoglobin-free-agar, but the characteristic bacillus does appear on the blood-agar controls, report the organism as *B. influenzae.* In making transplants from blood-agar to plain agar in the cultural test, great care must be exercised to prevent carrying over hemoglobin-containing medium. Often a small trace of hemoglobin will be sufficient to permit growth of *B. influenzae* on the plain agar. A transplant from the first to a second plain agar slant may be necessary to determine the diagnosis.

For B. tuberculosis. To make smear of sputum always use new slides which have been well cleaned. With two platinum wires which have been well flamed pick out portions of the sputum. If these are present, select "rice bodies"; avoid clear mucus and pure pus or blood. Make moderately thick smears; label slide with blue pencil; and allow smear to dry in air. Fix by passing slowly three times through the flame. Stain by the Ziehl-Neelsen method (p. 108) and examine for the characteristic red stained bacilli.

Cultures may at times be successfully made by treating the sputum with antiformin (p. 125), and separating and transplanting residue to Lubenau's egg medium. The culturing of the organism is rarely ordered.

For animal inoculation, select masses of sputum or "rice bodies"; wash through series of Petri dishes containing physiological salt solution. Transfer the washed portions to a strong sterile test-tube; add about 1.5 to 2 c.c. sterile salt solution, and emulsify by rubbing with a sterile glass rod. Inject subcutaneously into the inguinal region of a normal guinea-pig, (p. 113).

For Diplococcus pneumoniae. Make two smears on clean glass slides; dry in air and fix in flame. Stain one by the Gram method and the other for capsules by the Hiss capsule stain. Examine for characteristic organisms.

If cultures are ordered for isolation of the organism, wash portions of the sputum as outlined above (under *B. influenzae*), and transfer to either blood-agar slants or Loeffler blood serum medium, and to dextrose broth tube. After growth develops, stain by Gram method, and if characteristic organisms are present, plate from dextrose broth culture with plain agar in the usual way. From growth on plates after twenty-four hours' incubation, fish small bluish-gray colonies and inoculate dextrose broth tubes. If Gram stain shows organisms morphologically resembling the pneumococcus, inoculate one litmus milk and two inulin-serum water tubes each with 3 to 5 drops of the broth culture by means of sterile Pasteur pipette. In the absence of any coagulation of the inoculated media incubate for three days before making final report. Make smears from litmus-milk culture if it shows coagulation, and do capsule stain.

For method of determining group classification of pneumococci, see p 134.

For animal inoculation, wash the sputum in the usual way, prepare emulsion, and inject into mice (p. 135).

EXAMINATION FOR B. TUBERCULOSIS.

Antiformin method for separating B. tuberculosis. By the use of antiformin[1] tubercle bacilli may be freed from other materials and concentrated for examination or inoculation. It is very necessary in the handling of the suspected materials to use glassware which has been thoroughly cleaned, and water for dilution and washing which has been freshly distilled and collected in perfectly clean flasks. Otherwise, certain acid-fast bacteria may enter as contaminants, and give rise to error in diagnosis. Dilute sputum or other fluids to 40 c.c. in a bottle, and mix with 8 c.c antiformin; close with a *clean* rubber stopper, and shake occasionally until the mucus, etc., is dissolved.

In the case of pus, caseous material, minced tissue, etc., add an equal volume of antiformin[2], shake occasionally until the material becomes dissolved, then add several volumes of distilled water. Solution may be hastened by placing the bottle in an incubator at 37 C. for some time. When the materials have gone into solution, pipette into clean tubes, and throw down sediment in centrifuge. Remove supernatant fluid with pipette, and disinfect. Add distilled water; mix; centrifuge again; and repeat the process several times to wash out the antiformin. Finally, from the residue make smear on clean slide; fix by heat; and stain by the Ziehl-Neelsen method. It may be necessary to smear the slide with a very little egg albumen as a fixative before spreading the sediment upon it, but as a rule this is not necessary if the sediment is well washed. If desired, animal inoculations and cultures on Lubenau's egg medium may be made.

A modification of the above method consists of dissolving sputum in the antiformin, then adding ligroin (sp. gr. 0.715 to 0.72) to the solution, and shaking thoroughly. This mixture is then centrifuged, during which process the organisms collect in

1. According to Rosenau, this consists of equal parts of liquor sodæ chlorinatæ, and a 15 per cent. solution of caustic soda. The liquor sodæ chlorinatæ is prepared as follows:

Sodium carbonate	600
Chlorinated lime	400
Distilled water	4000

2. If the antiformin is good, a foaming should take place when it is added to the material and shaken.

a layer between the dissolved sputum and the ligroin. Materials for examination may be removed by means of a capillary pipette, fixed on clean slide, and stained in the usual way. This method is used when only microscopical examinations are required.

All glassware, etc., used in the above procedure must be sterilized, since the tubercle bacilli are not killed by the antiformin.

Spinal fluid. Place the tube containing the fluid, as soon as it is drawn, in the incubator until a delicate clot is formed. Carefully spread out the clot on a clean new slide; fix by heat; and stain in the usual way. If no coagulum appears after four or five hours, scrub finger; prick with needle; and allow a small drop of blood to fall into the tube. This is often found to be efficient.

Stools: Make a watery emulsion of the stool, and centrifuge in clean tubes at moderate speed for ten minutes to remove coarse particles. Remove supernatant fluid to a clean tube and dilute with equal volume of 95 per cent. alcohol; then centrifuge for one half hour or more. From sediment make smears on clean new slides; fix in heat; stain with hot carbol-fuchsin and decolorize in Pappenheim solution. Disinfect the glassware after using.

Urine. Dilute the urine with equal volume of 95 per cent. alcohol, and centrifuge at high speed in sterile clean tubes for one-half hour. Remove supernatant fluid to a clean tube (disinfect); make smears from sediment on clean new slides; stain with carbol-fuchsin in the usual way; and decolorize with Pappenheim's solution. It is well to moisten slide, before making smear, with serum or egg albumen, to fix the bacilli if present.

EXAMINATION FOR TREPONEMA PALLIDUM.

Collection of material. Examinations are usually made of serum from suspected primary lesions, although the treponema may be found in scrapings from secondary lesions. Wash the surface of the ulcer to remove extraneous organisms, then rub with a piece of dry gauze until slightly abraded, but not so that blood exudes to any extent, as this obscures the field. Express serum by squeezing the indurated area, or aspirate with a small Bier cup. If bleeding occurs, wait a few minutes until it stops; then, not infrequently, clear serum may be obtained.

Methods of examination. "Dark Field." Put a drop of the collected serum on a clean thin slide and place over it a cover-

slip, avoiding the inclusion of air bubbles. Put a drop of immersion oil on the under surface of the slide, and place it on the dark field stage also without including air bubbles. Examine with high power dry lens. Illuminate the stage by focusing the light from an arc light on the plain mirror by means of a large condensing lens, and throw out the Abbe condenser.

India ink method (*Burri*). Place a small drop of fresh "Chin Chin" ink upon a clean slide. Collect the serum from the chancre by passing the end of a slide across it; place the serum in the drop of ink; and draw along the slide to make a thin film (ink and serum), as in blood smears. When dry, examine with oil immersion objective for organisms which appear white in a brown or black field.

Wood stain. Make smears by drawing the flat surface of a clean slide across the lesion, or collect a drop of serum on the end of a slide and make a smear similar to a blood smear. Fix preparation 3 minutes in methyl alcohol, then proceed to stain by the same method as that used for blood smears. Place for one minute in eosin solution; drain; cover with methylene azure; and allow to remain for about 3 minutes before washing.

PREPARATION OF BACTERIAL VACCINES.

Culturing Organisms. Before seeding medium, each organism must be separated, and grown in pure culture. Use plain agar slants in tubes or flasks for seeding of such organisms as the staphylococcus group, the *B. coli* and *B. typhosus* groups, etc.; use dextrose broth in flask for the pneumococcus and streptococcus groups, also for the *M. catarrhalis* group; for *B. influenzae* use 5 per cent. blood-agar slants and for the gonococcus use dextrose ascitic-agar slants or starch-agar slants.

Apparatus Required. For the preparation of a vaccine from a bacterial culture, the following articles and materials are necessary: sterile, plugged 1 c.c. pipettes, graduated in 0.01 c.c.; sterile, plugged 5 c.c. bulb pipettes; sterile, plugged 10 c.c. graduated pipettes; sterile plugged Pasteur pipettes 20 cm. long, 2 to 3 c.c. capacity; sterile test-tubes (1.5 x 15 cm.) containing 8 to 10 small beads (2 or 3 mm. diam.); vaccine centrifuge tubes (Hopkins model), which have been plugged with cotton covered with two to three layers of gauze, then placed in metal container, and sterilized by dry heat; filters for bacterial suspension (these are

usually prepared from vaccine centrifuge tubes with broken tips, by loosely packing the bottom with a layer of absorbent cotton 3 to 5 mm., placing the whole, tip downward, in a large test-tube; plugging; and sterilizing by dry heat); clean bottles (10 to 15 c.c.) containing 8 to 10 small beads, which have been plugged with cotton covered with a layer of gauze; then placed in a metal container and sterilized by dry heat; sterile rubber stoppers for bottles.

It is essential to have a water bath with water to the depth of 15 cm. in which a constant temperature can be maintained. The models which are used in chemical laboratories give satisfactory results. In addition, a large metal tripod, a Bunsen burner, and an accurate thermometer are necessary.

Solutions Required: Sterile physiological (0.85 per cent.) salt solution made in the usual way; 0.25 per cent. tricresol, made by adding 0.5 c.c. cresol by means of a sterile 1 c.c. pipette (plugged) to a sterile bottle, and to this 200 c.c. sterile distilled water, sealing the bottle with a sterile rubber stopper, and shaking mixture until cresol is dissolved; 8 per cent. mixture of cresol made in distilled water by placing, with a sterile 1 c.c. pipette, 1 c.c. cresol in a small sterile bottle, and adding 11.5 c.c. sterile distilled water, sealing bottle with sterile rubber stopper, and immediately before withdrawing portions for use vigorously shaking mixture to emulsify it.

Procedure. This is carried out in a clean quiet room, free from dust. After seeding medium, allow to grow at incubator temperature for about 24 hours; then make Gram stains and examine for purity before proceeding. If the culture proves to be pure, in the case of solid medium add a sufficient quantity of sterile salt solution (with a sterile 10 c.c graduated pipette) to make a heavy suspension after washing off the organisms. Place a vaccine centrifuge tube in test-tube stand, remove the plug, and place one of the filters in the mouth of the tube. With a Pasteur pipette* take up the salt solution suspension of organisms and strain into the vaccine centrifuge tube through the cotton in the filter until about 10 c.c. are carried over. In the case of cultures in dextrose broth, remove material from the bottom, and filter

*Always use a small rubber suction bulb on end of the pipette to handle fluids.

into a vaccine centrifuge tube. After transferring the suspension of organisms, place pipette in cylinder of cresol solution on worktable to disinfect. Remove filter, and place in boiler for sterilization. Immediately return sterile plug to vaccine centrifuge tube; bend down free end over edge, and fasten with elastic band; then secure by passing a small strip of adhesive across the top of plug and fastening it down at sides of tube. Label tube with patient's name and type of organism. Counterpoise tube and place in centrifuge. Throw down and pack the bacteria by running machine at the sixth speed for 30 minutes; then with Pasteur pipette remove supernatant fluid (disinfect) and the bacterial sediment to the 0.05 c.c. mark, then add with a sterile 10 c.c. graduated pipette sufficient sterile physiological salt solution to bring volume to 5 c.c. for a 1 per cent. suspension. If less than 0.05 c.c. residue is recovered, add salt solution as necessary to give a 1 per cent. suspension. After adding the salt solution, make a uniform mixture by drawing the fluid and organisms in and out with Pasteur pipette.

Killing Organisms. For this purpose, remove the bacterial suspension from the vaccine centrifuge tube* and place in the sterile test-tube containing the beads. Shake until a perfectly homogeneous suspension results, taking care not to allow the fluid to reach the top of tube or touch plug. When a uniform fine suspension is obtained, remove the plug (place in boiler); sterilize in flame of burner the open end and 4 to 5 cm. below; then take a fresh plug from a sterile test-tube of same size and close. In the meanwhile heat the water in the bath, and bring it to a temperature of 60 C. Submerge the test-tube containing the bacterial suspension, upright in the water bath so that the water level rises within about 2 cm. of the end. Heat the suspension at a constant temperature of 60 C. for one hour.

Sterility Controls on Vaccine. Remove the tube from the water bath after heating is finished, and transfer vaccine to one of the small (10 or 15 c.c.) sterile bottles with a sterile 5 c.c. bulb pipette. Make three control inoculations from vaccine prepared from organisms grown on ordinary medium by adding, with a sterile Pasteur pipette, one drop to an agar slant, one drop to a plain broth tube, and two drops to a flask of plain broth such as is

*When empty, sterilize by boiling.

used for blood cultures. In the case of organisms grown on dextrose broth, dextrose media are used for inoculation of controls, and for organisms growing only upon special media the controls must be made on the same medium. Incubate the inoculated media for three days; if no growth occurs in this period the vaccine may be accepted for use. After making the controls, add tricresol to the vaccine in the same bottle. Vigorously shake the bottle containing the 8 per cent. tricresol mixture until it is emulsified, immediately withdraw 0.25 c.c. by means of a sterile 1 c.c. pipette (plugged) and add to the vaccine (about 5 c.c.) in the small bottle. Securely seal the vaccine bottle with sterile (boiled) rubber stopper; shake thoroughly to put tricresol in solution; then cap stopper and neck with tinfoil, and fasten by passing a strip of adhesive over the top and attaching at sides of bottle. Label the bottle with patient's name, species of organism, volume and concentration (1 per cent.) of suspension, and date; then place in the ice box in a dark place.

Standardization of Vaccine. The standardization is based upon the table of Hopkins for averaged 1 per cent. suspensions prepared as just outlined for the following organisms:

Staphylococcus aureus and albus	=	10	billions	per c.c.
Streptococcus hæmolyticus	=	8	"	" "
Gonococcus	=	8	"	" "
Pneumococcus	=	2.5	"	" "
Bacillus typhosus	=	8	"	" "
Bacillus coli	=	4	"	" "

Dilutions of Vaccine for Use. The dilutions are made from the 1 per cent. stock suspension. To prepare a given dilution, determine the volume of stock solution which contains the desired number of organisms to make the quantity ordered. Measure this amount by means of sterile 1 c.c. plugged pipette; place in small sterile bottle; and add sufficient volume of 0.25 per cent. tricresol solution to bring to required dilution.

Vaccines for intravenous injection are diluted in the same way, except that sterile physiological salt solution must be used as a diluent instead of the tricresol solution. Seal bottle with sterile rubber stopper; cap with tinfoil; and fasten with a strip of adhesive. Label with patient's name, species of organism, number of organisms in each c.c., and date. All vaccines and dilutions should be kept in ice box.

Each week the hospital milk (both the certified and the loose) are examined bacteriologically. As a routine, the samples are taken by the bacteriologist early in the morning on test days, and platings are made at once. The plates are allowed seventy-two hours for the development of colonies before the counts are made.

Apparatus and Materials Required. Prepare a number of "water blanks" for dilution purposes as follows: Wash thoroughly a number of square bottles (8 oz.), and dry. Tightly plug each with a stopper made of ordinary cotton covered with cheese cloth, then sterilize with dry heat. To each bottle add 100 c.c. distilled water and mark upper level of fluid with a small adhesive strip, then add 8 c.c. excess to each to compensate for loss by evaporation during sterilization. Cap each with white paper, place in autoclave and heat at 15 lbs. pressure for 30 minutes. Store and use as required, but do not use if volume has been much altered by evaporation as indicated by adhesive strip marker on bottle. Wrap in clean, strong paper a number of Petri dishes in packages of three, and sterilize by dry heat. Plug with cotton a number of 1 c.c. (graduated in 0.01 c.c.) pipettes, place in copper containers and sterilize with dry heat. Prepare in the same way two or three 10 c.c. bulb pipettes. Clean, plug, and cap several large test-tubes (2.5 x 15 cm.); sterilize by dry heat. Sterilize by boiling several rubber stoppers which will fit the mouths of the water-blank bottles. Prepare plain agar tubes for plating and neutral-red lactose peptone fermentation tubes. Cup for liquifying media, and thermometer for regulating the temperature for plating.

Taking of samples. The milk is procured from the steward. When it is possible to mix milk thoroughly without outside contamination, this should be done; otherwise, a sample below the cream zone is taken. Take sterile capped test tubes and container with sterile 10 c.c. bulb pipette to steward's ice-box. After the cover has been removed from the can, place finger over upper end of pipette and plunge pipette quickly downward to a depth of about 25 cm. (10 inches). Draw sample; place in one of the sterile test tubes; plug; and take to the laboratory. Also get a bottle of certified milk with seal intact.

Procedure. After reaching the laboratory, the tests must be

carried out immediately in a quiet room, on table which has been washed free from dust. Shake thoroughly the sample of milk in the test tube; remove 1 c.c. with sterile 1 c.c. pipette; and add to 100 c.c. water blank. Seal with a sterile rubber stopper, and shake vigorously for 3 to 5 minutes. If necessary make a second dilution by removing 10 c.c. of water by means of sterile pipette from water blank, then add 10 c.c. from first dilution, plug with rubber stopper, and shake thoroughly. Arrange two series of Petri dishes on the cleaned table; if the first dilution only is to be plated, put three dishes in each series; if both dilutions are to be used, six dishes to each series. By means of a sterile 1 c.c. pipette take 1 c.c. of the first dilution (1:100), raise cover of first Petri dish, and carefully measure in the amount as near the center as possible; in the same way add 0.5 c.c. to the second dish, and 0.2 c.c. to the third. If the second dilution (1:1000) is used, take another sterile pipette and proceed as with the first. In the meanwhile, liquify plain agar tubes by boiling, and cool to 42 C. To each dish add the contents of agar tube directly to the measured milk dilution, and agitate quickly to insure full mixing and spreading of the medium. Before pouring the medium, remove plug, and in free flame sterilize the lip and 6 to 8 cm. below. The second series of plates is carried out in duplicate as outlined. Label each plate with name of milk, the quantity, the date, and the temperature to be used for incubation. Invert plates, stack each series, and fasten together by a strip of adhesive tape. Place one series in the incubator at 37 C., the other in a dark closet at room temperature, to develop. After seventy-two hours, count the number of colonies which have developed on each. Also inoculate a series of neutral-red lactose peptone fermentation tubes with the milk to test for gas-forming organisms. To the first tube add 1 c.c. undiluted milk; to the second 0.1 c.c. undiluted milk; to the third, 1 c.c. of a dilution of 1:100; and to the fourth, 0.1 c.c. of a dilution of 1:100. Place these tubes in incubator at 37 C. If gas forms, make platings on Endo's medium, fish pink colonies and inoculate on plain broth tubes. Make Gram stains from cultures, and if Gram-negative bacilli are found, place on sugar fermentation tubes to determine types.

In handling the certified milk which is received in original bottles, shake very thoroughly and then remove outer cap. Note

source (dairy farm), date of bottling, and the medical society certifying the milk, all of which data appear on the inner seal. Wipe mouth of bottle and inner cap with clean towel moistened with water, care being taken that towel is sufficiently dry to permit no droplets to remain on bottle or cap. Make dilutions, and proceed in the same way as outlined above.

Counting Colonies. After seventy-two hours incubation, the colonies which have developed are counted on each plate. In general not much difficulty is experienced in determining the number of colonies on a plate made from certified or pasteurized milk. If the colonies are not greatly crowded together, the bottom of the plate may be marked off into fields by drawing lines from the center to the periphery, and counting and recording the number of colonies in each field. Add these totals to get total number on plate and multiply by dilution and volume to get number of colonies per c.c. After making these determinations for each plate, average them and report the average. Time may be saved by using Wolffhügel's colony counter on plates which show numerous colonies evenly distributed. Invert the Petri dish, place the counting plate over it, and count the number of colonies in each of ten of the ruled squares (1 cm.). Take the average for one square, then determine the area of the plate*; and from this calculate the approximate number of colonies on the plate. From this number estimate the number per c.c. of milk, taking into consideration the dilution and the volume plated. Record in bacteriological record book all the data with results of the milk tests.

*Rule: Multiply the square of the radius by 3.1416; the product will be the area required.

SEROLOGICAL TECHNIQUE

DIFFERENTIATION OF TYPES OF PNEUMOCOCCI.

According to Dochez and Gillespie, pneumococci causing acute lobar pneumonia may be classed in four general groups, by means of specific agglutinating sera, certain morphological characteristics, etc. Cultures may be obtained from blood, sputum, etc. Sputum is the material most commonly submitted for examination; therefore, the method of testing this, based upon that originating in the Rockefeller Institute Hospital, will be outlined accordingly.

Apparatus and Materials Required. Sterile Esmarch dishes; sterile small porcelain mortar and pestle; sterile plugged test-tubes (1 x 10 cm.); sterile plugged conical 15 c.c. centrifuge tubes; sterile plugged Pasteur pipettes (18 to 20 cm. long, 2 to 3 c.c. capacity); sterile plugged 1 c.c. pipettes, graduated in 0.01 c.c.; sterile all-glass 2 c.c. syringe with hypodermic needle (2.5 cm. long, 20 gauge); battery jar with cover (for mouse); clean glass slides; metal test-tube stands; water bath (37 C.); sterile instruments (small scissors, scalpels, forceps, etc.) for autopsy; instrument boilers; sterile physiological salt solution; specific agglutinating sera (Groups I and II); sterile ox bile; 95 per cent. alcohol; 5 per cent. carbolic acid solution; culture media (plain broth, blood-agar slants, or plates); white mice for inoculation.

Preparation of Sputum. Collect the sputum in sputum cups, and, if possible, get the portion from the deeper air passages. Make direct smears of the sputum on clean slides; stain by Gram method, also by Hiss capsule stain. If organisms resembling pneumococci are present, arrange and partially fill a series of three or more sterile Esmarch dishes with sterile physiological salt solution. By means of platinum wires fish from the sputum one or more of the denser masses about the size of a bean, and place in the first wash dish. Wash the materials through the series of dishes containing salt solution, handling and manipulating with sterile platinum wires. Transfer the thoroughly washed

masses[1] to the sterile mortar and by means of pestle rub into a homogeneous emulsion, adding sterile salt solution (or bouillon) drop by drop until mixture is sufficiently diluted to pass readily through the syringe needle.

Inoculation of Mouse. Draw 1 or 1.5 c.c. of the emulsified sputum into the sterile syringe through the needle. Select a healthy white mouse for intraperitoneal inoculation. Grasp the animal by the tail with the right hand, then, while gently pulling backwards, grasp the nape of the neck with the thumb and forefinger of the left hand, extend the animal (back to palm of hand) and hold in position for injection by pressing tip of third finger of left hand over root of animal's tail against the base of the thumb. With the freed right hand wipe skin over abdomen with 95 per cent. alcohol on a cotton wipe. Take loaded syringe; puncture the skin in the lower part of abdomen with needle; pass point upward between skin and muscular wall about 2 cm., then puncture the wall and inject the emulsified sputum into the peritoneal cavity. Withdraw needle; place syringe in boiler; wipe skin at point of puncture with 5 per cent. carbolic acid solution on cotton wipe. Place the mouse in the animal jar, which should contain cotton for bedding, also food and water if the animal is not to be killed within a few hours. Label the jar with patient's name, time of injection of animal, and date.

Autopsy of Mouse. In case an early diagnosis is desired, kill the mouse 6 to 8 hours after the injection; otherwise do this later, or await death of the animal. Fasten the dead animal, back downward, to a board by extending legs and placing tacks through the feet. Place in large tray for autopsy. Wash skin over the body with 5 per cent. solution carbolic acid; wipe partially dry with cotton wipe. Lift skin with sterile forceps; make cut just through skin in lower abdominal area; and with sterile scissors extend incision up the median line to the neck region. Dissect back skin on each side with sterile scalpel, exposing the body wall. With sterile forceps lift abdominal wall: make a small incision through wall; and, by means of sterile platinum loop, remove material and make cultures on blood-agar slants and plain broth; then make smears on clean slides. Note the character of the peritoneal exudate; observe especially if it is muci-

1. Cultures may be made also from the washed material if desired.

nous, stringing out on removing platinum loop. Stain the smears by Gram method, and Hiss capsule stain. If numerous characteristic organisms are present, open the abdominal cavity fully; wash out the exudate with 8 to 10 c.c. sterile physiological salt solution; and place it in a sterile 15 c.c. conical centrifuge tube. Wash the cavity by taking about 1 c.c. of the salt solution in a Pasteur pipette with rubber suction nipple, lifting one side of abdominal wall, and discharging the salt solution into the cavity. Draw the fluid back and forth until it becomes quite turbid, then take up and transfer to centrifuge tube. Repeat the process until the cavity and the contained viscera are thoroughly washed. Disinfect the pipettes. After the peritoneal exudate has been removed, open the thorax, under aseptic precautions, and expose the heart. Make an opening to cavity with sterile scissors, then make culture from heart blood on to blood-agar slants and plain broth. Cover body with layer of cotton wet with 5 per cent. carbolic acid, and place, without removing from board, in covered can for cremation. Sterilize all instruments by boiling.

Preparation of Bacterial Suspension. Fasten the plug securely in the mouth of the 15 c.c. centrifuge tube containing the washings from peritoneal cavity of the mouse. Counterpoise and place in the centrifuge, run machine at low speed for 3 to 4 minutes, to throw down leucocytes, etc. Transfer the slightly turbid fluid to a second sterile 15 c.c. conical centrifuge tube; fasten in plug; counterpoise, and place in centrifuge. Throw down the organisms by running machine at sixth speed for 20 to 30 minutes. Remove and disinfect the supernatant fluid, leaving the bacterial residue, to which add sufficient physiological salt solution to give a turbidity about equivalent to a good twenty-four hour broth culture of the pneumococcus.

Agglutinating Serum Test. Place in metal test-tube stand six plugged small sterile test-tubes (1 x 10 cm.). Into each of the first two tubes place 0.3 c.c. of specific agglutinating serum for Group I; into each of the second two, the same quantity of specific agglutinating serum for Group II; in the fifth tube 0.1 c.c. sterile ox bile, and in the last tube, 0.3 c.c. physiological salt solution; finally, add 0.3 c.c. of the homogeneous bacterial suspension; plug tubes; mix by gently shaking; and place in water bath at 37 C. Incubate for two hours, then place in ice-box over night if no definite agglutination has occurred.

Reading and Interpretation of Results. Agglutination when present may be quite variable as to time of appearance and quantity. Occasionally this reaction occurs very quickly, even before the tubes are placed in the bath; other cases vary from first to second hour of incubation even after standing over night in the ice box. The reaction is generally easily recognized by the flocculi of various sizes which settle more or less quickly to bottom of the test-tube. Readings must be checked against the control suspension in sixth tube. The bacterial suspension (pneumococcus) should become clear in the tube containing the ox bile. In case the pneumococcus is overgrown by other organisms in the peritoneal exudate, the test may become impracticable. However, the broth culture made from heart blood of mouse at autopsy may show sufficient growth of the pneumococcus, so that the test can be carried out directly with the culture.

If properly controlled tests show agglutination with either specific agglutinating serum for Group I or Group II, the results are reported accordingly. When no agglutination occurs with either serum, the organism is classed Group IV when the stains made from peritoneal exudate or cultures show that it is not *Pneumococcus mucosus capsulatus.* This organism is classed as Group III. After readings are made from tests, sterilize the test-tubes and contents by boiling 15 minutes in water.

PREPARATION OF SALVARSANIZED SERUM FOR INTRASPINOUS INJECTION.

Preparation of Glassware. With soap and water wash thoroughly large mouthed 8 oz. square bottles, and large mouthed (Rockefeller Institute model) 50 c.c. centrifuge tubes. Rinse in running water; then fill with sulphuric acid-potassium bichromate mixture, and allow to stand over night. Empty cleaning mixture into stock bottle, rinse in running water several times, then wash in stream of flowing water for at least two hours by passing tube to the bottom of each container and allowing the water to overflow at top. After washing rinse three or four times with a liberal amount of distilled water; drain; dry; then stopper mouths with plugs made of cotton wrapped in about four layers of cheese cloth. Trim the free end of the plugs smooth, and cap so as to cover the plug and neck of the container fully. In the case of bottles use strong white paper; for centrifuge tubes,

use clean muslin. Sterilize by dry heat for one hour at 170 to 180 C. After sterilization label each bottle with printed form, and reserve for ward use.

Taking of Blood and Separation of Serum. The blood is drawn from the patient by the ward physician under aseptic precautions, and placed in the specially prepared bottle, which is carefully stoppered. Care should be taken to use a dry syringe and needle in taking the blood, and in emptying the syringe not to apply unnecessary force, nor allow the blood to form foam with air bubbles, which may result in marked hemoglobin staining of the serum. The bottle containing the freshly drawn blood is inclined at a slant, care being taken to prevent the blood from touching the stopper, and coagulation is allowed to take place. After a firm coagulum is formed, the bottle is carried in the same position and placed in special holder in the ice-box in the bacteriological laboratory where it generally remains over night. Usually the serum separates well from the clot, but if it does not, loosen clot from walls of bottle with sterile wire, and allow to stand at room temperature for about one hour, until separation does take place.

Preparation of Serum. When the serum has well separated take bottle to a closed quiet room, where the work-desk has been well washed off with 1:1000 solution of bichloride of mercury. Remove the serum from the clot by means of a clean sterile 25 c.c. pipette which has been well plugged with cotton. The serum may be drawn up by mouth suction or with a rubber bulb attached. Place the serum in one or two of the specially prepared and sterilized 50 c.c. centrifuge tubes. Turn the free margins of the plug over lip of the tube and tie securely with a strong thread or cord. Place the tube in centrifuge and run at third or fourth speed until cells are entirely sedimented, which usually requires about one-half hour. Stop the machine by "stepping down" gradually, finally shutting off the switch after 3 to 5 minutes. Often cells will rise if the machine is stopped too quickly. When the cells are entirely separated from the serum, take the tube to the quiet room, and remove the serum by means of a sterile 10 c.c. pipette, (straight, graduated) with rubber bulb, and measure into a second sterile tube of the same kind. Exercise great care in removing the serum to allow no back flow, or any other manipulation which may cause stirring

up of the sediment at the bottom. In case it is not desired to use the serum full strength value, add sufficient sterile physiological salt solution to give the percentage value of serum ordered. Plug the tube securely, cap with tinfoil, care being taken to keep the lower margin of the foil above the water level when tube is placed in water bath. Finally, place the tube in a water bath, and heat at 56 C. for one hour; the level of the water should never be below that of the serum; if possible it should be kept above. After the serum has been heated at the required temperature for the required time, remove from the bath, put on label with patient's name, ward, percentage value of serum, and date; then place in the ice-box until sent for from ward. In case the serum is deeply stained with hemoglobin, notify the ward physician who has the case in charge before carrying out the final heating, and get instructions whether to proceed or not.

PREPARATION OF NORMAL SERUM FOR SUBCUTANEOUS INJECTION.

The procedure in this case is the same as that outlined under Preparation of Salvarsanized Serum, with the exception that the final step of heating the serum is omitted in this instance, unless ordered by the physician in charge of the case.

BLOOD TESTS FOR TRANSFUSION CASES.

(Hemolysin and Hemagglutinin)

Apparatus Required: Sterile, plugged 1 c.c. pipettes graduated in 0.01 c.c.; sterile 15 c.c. graduated conical centrifuge tubes for separation of serum, washing of corpuscles, etc.; sterile test-tubes (1.5 x 15 cm.) containing about 8 to 10 glass beads (2 or 3 mm. diameter) for defibrinating blood; small glass funnels (3 or 4 cm. diameter); sterile gauze (5 x 5 cm.) to strain defibrinated blood; sterile test-tubes (1.25 x 10 cm.) for serum; sterile test-tubes (2.5 x 10 cm.) for collection of blood for serum; sterile test-tubes (1 x 10 cm.) for test (these tubes must be specially washed in soap and water, rinsed in running tap water; placed in 5 per cent. hydrochloric acid for some time; washed in running tap water; rinsed 2 or 3 times with distilled water; and then dried. The glass should be transparent and show no cloudiness); suitable stands for test-tubes.

Taking of Blood. Draw blood from vein in arm of prospective

donors and of the patient, with a clean, perfectly dry syringe From each donor take about 10 c.c.* of blood, put 1 or 1.5 c.c. in tube containing beads, shake tubes about 15 minutes to defibrinate blood; empty the remainder of the blood into large test-tube; and slant for coagulation and separation of serum. Draw blood from the patient in the same way, controlling the amount in accordance with the number of tests which are to be done. For six donors, defibrinate 1 or 1.5 c.c., and clot about 7 or 8 c.c. for serum. Take history and fill out card for each donor and the patient.

Preparation of Blood. (a) *Serum.* Draw off the serum with Pasteur pipette from the clot as soon as separated, place in 15 c.c. centrifuge tube (labelled with individual's name on strip of adhesive), and throw down cells in the machine. Remove supernatant serum, place in test-tube, and store in ice-box until the test is made.

(b) *Blood cell suspension.* In each case add a few c.c. of physiological salt solution (0.85 per cent.) to the defibrinated blood, and mix. Place 2 or 3 layers of the sterile gauze in a small funnel, through which strain the blood suspension into a 15 c.c. graduated centrifuge tube (label with individual's name); wash blood from defibrinating tube with a second portion of salt solution, and pass through gauze; remove funnel, and add sufficient salt solution to bring total in the tube up to 15 c.c. or more. Throw down the cells in the centrifuge at fourth or fifth speed for 3 to 5 minutes. Remove the supernatant fluid (disinfect), then add the same volume of fresh salt solution, mix by drawing back and forth in Pasteur pipette by means of rubber bulb. Again throw the cells down, and remove the supernatant fluid. Finally, add salt solution again; mix thoroughly; place in centrifuge, and run machine at third speed for 15 minutes to wash and pack cells. Remove supernatant fluid and in case of donors, all corpuscles except 0.2 c.c. (read by graduation marks on tube), then add salt solution to bring volume up to 10 c.c., which on mixing gives a 2 per cent. suspension of corpuscles. Wash patient's blood in the same way, and prepare a 2 per cent. suspension of corpuscles with the salt solution, in such quantity as to cover

*This gives sufficient serum for the tests, including the Wassermann reaction.

fully the required number of tests, based on the number of possible donors.

Procedure. For each donor set up in stand three series of four tubes each, and two extra tubes for blood suspension controls, using the small, specially prepared test-tubes. Mark the tubes in each series as follows:

1st Pc (Patient's cells + Patient's serum).
Ps

2d Pc (Patient's cells + Donor's serum).
Ds

3d Dc (Donor's cells + Patient's serum).
Ps

4th Dc (Donor's cells + Donor's serum).
Ds

Mark the tubes for blood suspension controls either D (donor) or P (patient) as necessary.

The components are combined in each series as indicated above. To the first series add 0.25 c.c. of the respective sera to each tube, likewise the same amounts of 2 per cent. blood suspension; to the second series add 0.1 c.c. serum, 1.5 c.c. salt solution, and 0.25 c.c. blood suspension; to the third series add 0.05 c.c. serum, 2.0 c.c. salt solution, and 0.25 c.c. blood suspension. To the first of the blood suspension control tubes, add 0.5 c.c. of the 0.2 per cent. suspension of corpuscles; to the second tube add 0.25 c.c. of the suspension and 0.25 c.c. of physiological salt solution. In adding the serum, place tip of pipette in bottom of tube and measure in required amount. Always have a uniform suspension of the corpuscles before pipetting to the tubes. After the components are combined, shake tubes thoroughly, and place in bacteriological incubator at 37 C.

Readings. After one hour's incubation examine tubes for reaction. Compare color tint (hemolysis) by holding first and third and second and fourth tubes in each series side by side towards good light. Then slant each tube and observe whether the sedimented cells flow, or not, indicating absence or presence of agglutination. Do not shake tubes; return to incubator for a second incubation of one hour. At the end of this time, make comparative readings for any hemolysis which may be present: then examine the sedimented cells for agglutination by first slant-

ing and then shaking. Note on record cards the results of each reading as made. Place the tubes in the ice-box over night and on the following morning make a reading, which often brings out a delayed agglutination. The following markings are used to indicate the degrees of hemolysis: c (complete); ac (almost complete); p (partial); sl (slight); o (none). Agglutination is indicated by vm (very marked) adherent mass; m (marked) large clumps; rm (rather marked) small clumps; sl (slight) very fine clumps.

RAPID METHOD OF TESTING BLOOD FOR TRANSFUSION CASES.

Rous and Turner have published a method which may be quickly performed and is especially applicable to emergency cases. Their method is based in particular on the hemagglutinin reaction, and the readings are made microscopically.

Apparatus and Materials Required. 1:10 blood mixing pipette (leucocyte type) with capacity of about 0.25 c.c.; capillary pipettes (Wright's); clean slides and cover-slips; small narrow test-tubes; small mixing cups or hollow ground slides; microscope; 10 per cent. sodium citrate solution in distilled water; physiological (0.85 per cent.) salt solution.

Collection of Blood. Rinse blood-mixing pipette with 10 per cent. sodium citrate solution; empty; then draw solution up to mark 1 on the pipette; fill with blood from finger or ear puncture; and expel at once into small test-tube. If flow stops before pipette is filled, expel at once into tube to mix; make new puncture; take up mixture; and draw in required amount of blood. Pressure may be used to cause flow of blood. After taking blood, wash pipette with citrate solution, then with distilled water; rinse with citrate solution; take up the required amount of fresh citrate solution; and proceed to next case.

Mixing Blood Suspension. Make two combinations of the patient's blood with each donor's blood from the citrated blood suspensions; first, 9 parts of patient's blood to 1 part of donor's; second, 1 part of patient's blood to 1 part of donor's. In emergency only the first mixture need be made. Measure by making a mark on capillary tube of pipette, draw blood to that point, then take in air bubble, and blood again, etc., until the required amounts are drawn up. Expel into mixing cup to insure full mixing;

then draw all up into pipette again; seal tip; and hold ready for examination.

Incubation. Keep the pipettes at room temperature, omitting the ordinary higher temperature incubation. It is better to make readings after 15 minutes, but when haste is necessary, they may be made in less time.

Readings. Break the capillary end of each pipette; express a small drop of the blood on to slide; superimpose a large drop of physiological salt solution without mixing; place cover-slip over the drop; and examine the preparation for agglutination under the microscope. In agglutination, the cells are stuck together in masses or irregular heaps, which must be distinguished from the rouleaux of normal blood.

WASSERMANN REACTION.

Among the numerous tests of the clinical laboratory none requires greater technical detail nor demands greater knowledge of the known facts of biochemical reactions than the so-called Wassermann reaction, which is based upon the phenomenon of complement fixation. The reagents entering into this reaction are of biological origin and their chemical compositions are little known. For reliable results, the reagents must be carefully prepared and accurately standardized. Various modifications of the original Wassermann technique have been applied, and, as a result, perhaps no two laboratories perform the test under the same conditions. All attempt, however, to conform with the recognized principles governing the complement fixation phenomenon, and its recognition by means of the hemolytic system as an indicator of the presence or absence of reaction.

The serologist, unlike the analytical chemist, has no definite chemical substance on which the standardization of his reagents may be based, and he must, therefore, look to some one of the several biological reagents available, which is practically constant in value. Serum complement may be used as the primary standard reagent, but the conditions governing its source, preparation, and use must be fixed. By means of complement, the hemolytic immune body (amboceptor of Ehrlich) can be standardized. The immune body is a fairly stable substance, and under certain conditions can be kept for weeks or even months with little or no deterioration in value. After standardization, it serves as a standard reagent for the testing of other samples of complement, a labile substance.

Attention will be directed first to the preparation and standardization of the substances entering the hemolytic system.

SHEEP CORPUSCLES.

Drawing of Blood. Sterilize, by boiling, a bleeding needle, preferably one about 8 to 9 cm. long, with a bore of 2 to 3 mm. Also sterilize, by dry heat, a blood-collecting test-tube measuring

2.5 x 15 cm., containing a small spiral coil of copper wire, and firmly plugged with cotton covered with double layers of cheese cloth, then capped with paper. While the sheep is held by an assistant, trim the wool, using curved scissors, just laterally to the anterior midline of the animal's neck over an area of about 8 x 10 cm.; then lather, and shave clean. Locate the jugular vein by palpation. Wash the shaved area with 5 per cent. solution of carbolic acid. Press the thumb of the left hand over the proximal end of the vessel, causing it to distend with blood; then with the right hand pick up the sterile bleeding needle with a piece of sterile gauze and puncture the vessel.*

Collect the desired amount of blood in the tube as the blood flows from the needle. Plug the tube, and defibrinate the blood by shaking ten to fifteen minutes. After the removal of the needle from the blood vessel, moisten the punctured point with 5 per cent. carbolic acid solution; if the bleeding persists, grasp the skin between the thumb and forefinger over the point, and pinch firmly for about half a minute to form a clot.

It is preferable for a laboratory to keep one or more stock sheep for a supply of blood; if this is not practical, blood may be obtained from an abattoir. In such cases, collect the blood from the severed vessel in sterile tubes; defibrinate, and proceed as outlined. Blood so collected is usually infected, and as a general thing is not safe to use for immunizing purposes. It has been suggested by some workers that the blood be partially sterilized by heating (60 C. for one-half hour), which will destroy at least most of the vegetative forms of organisms, and is said not to injure the antigenic character of the blood cells. Of course, spores are not killed and may be the cause of infection in the animal.

Preparation of Corpuscles. The washing of the defibrinated blood is carried out as follows: Add, with a sterile pipette, 10 to 12 c.c. of the blood to a sterile 50 c.c. centrifuge tube; fill the tube with sterile 0.9 per cent. salt solution; mix thoroughly with pipette, and fasten in plug. Place in the centrifuge and throw down the cells, after which remove the supernatant fluid by

*If desired, the blood may be drawn from the vein in a 20 c.c. all-glass syringe, discharged into the collecting tube, and defibrinated. The drawing of blood in citrated solution is practised by some, but the writer prefers the defibrinated blood.

means of a sterile pipette; add more salt solution and mix well with sedimented cells. Again place in machine and throw down the cells; then remove the supernatant fluid. In all, this procedure is carried out three times. Finally, remove about 5 c.c. of the sedimented cells; place in a 15 c.c. graduated, conical centrifuge tube; add about 10 c.c. of salt solution; mix thoroughly and fasten in plug. Place the tube in the machine and run at high speed for fifteen minutes to pack thoroughly. Remove the supernatant fluid and, if desired, make a reading of the total volume of washed blood cells. The washed, packed corpuscles are now ready for use, either for immunizing or for test purposes.

COMPLEMENT.

Since complement plays the leading role in the Wassermann reaction it becomes necessary to secure a relatively uniform product of high value. Experience has shown that under certain conditions this object may be fairly well accomplished. For this purpose the blood serum of the guinea-pig gives the best results. Serum complement must be used as the primary standard by which the unit value of rabbit immune body is determined. Therefore, in preparing serum for such standardizations, it is necessary to follow rigidly the directions outlined below.

Selection and Care of Animals. Select only normal animals weighing from 350 to 600 gm. Keep in roomy, dry cages or runs in well lighted and freely ventilated rooms. Feed regularly morning and night. Bleed for complement just before the regular feeding time. Keep newly acquired animals, which have been transported, some days before bleeding, to accustom them to their surroundings. Take the animals selected for bleeding from the run; place at once under ether, and bleed without subjecting them to any undue excitement.

Bleeding of Animal. Always place the animal well under the anesthetic by putting a few c.c. of ether on cotton in a small paper cone, or better, in a large-mouthed vaseline bottle which can be slipped over the animal's nose. Take the blood, either by aspirating from the heart, or by bleeding from vessels of the neck. If the heart method is used, shave the chest, and by means of a dry, clean, 10 c.c. all-glass syringe with medium-sized needle (gauge 20) puncture the chest wall close to the sternum through the second or third right interspace. Direct

the needle downward and backward toward the left for 1 to 2 cm. Blood can be easily withdrawn if the needle has entered the heart cavity. Small pigs (350 gm.) will withstand the loss of 4 to 5 c.c. and survive, and larger pigs, of relatively more; or if the animal is to be bled to death, the same size pig may yield 10 to 12 c.c. by this method. If the animal is to be bled to death, trim the hair from the neck of the etherized pig; shave clean, and with a scalpel make an incision about 3 cm. long, through the skin over the trachea. By blunt dissection with forceps expose both carotids; cut through with scissors; allow the blood to flow into a funnel, and collect in a clean, dry, 50 c.c. graduated cylinder. Another method may be used successfully where only a small bleeding is to be made and the animal is to be saved. Prepare the animal for bleeding as outlined above, but make the skin incision parallel to the trachea, and midway between that region and the lateral aspect of the neck. Expose the larger, more superficial vein; cut with scissors; and catch the blood in a clean, dry cylinder as above. When the desired amount is collected, wash the operation wound with 5 per cent. carbolic acid; approximate the edges of the skin and closely suture with catgut which has been well soaked with 5 per cent. carbolic acid solution; wash the closed incision with the carbolic acid solution and place the animal in a cage. Generally the operation wound heals with no difficulty, and several bleedings may be made at intervals of three to four weeks.

Separation of Serum. Allow the blood collected from the animals to stand at room temperature for twenty to thirty minutes until a firm clot is formed. Separate the clot from the wall of the cylinder by a sterile glass rod or wire, then after plugging the cylinder with cotton, place it in the ice box for some hours. Generally the bleedings are made in the late afternoon, just before the night feeding time, and the clotted blood is allowed to stand in the ice-box until the following morning. Then remove the serum from the clot by a pipette; place it in a 15 c.c. centrifuge tube, and throw the cells down. Remove the clear serum and place it in sterile test tubes.

Character of Serum. Occasionally a serum on separation appears quite turbid. Such a serum should be rejected, since it is not suitable for complement. If the serum shows the presence of considerable hemoglobin it should be rejected for the

same reason. A serum suitable for complement should be perfectly transparent and free from hemoglobin, although a slight trace may be permissible under certain conditions.

Complement for Standardizing Immune Body. As pointed out, the hemolytic immune body must be standardized (unit value) primarily by means of complement. To prepare such a complement the serum from five or more guinea-pigs must be pooled. The conditions previously outlined must be rigidly observed. Under such circumstances, different lots of sera so prepared are practically of the same complement value, and may be called "standard complement."

Standardization of Complement. Since it is not practical in most laboratories to bleed five or more guinea-pigs for each series of Wassermann tests, an immune body is standardized and used to standardize the blood-serum from one or more animals. For this purpose, dilute the guinea-pig serum 1:10 with 0.9 per cent. salt solution, and also make a dilution of the hemolytic immune body in 0.9 per cent. salt solution, so that each c.c. contains two units.* Prepare a 5 per cent. suspension of sheep blood cells, in the regular way. Set up a series of test tubes in a suitable stand, and combine the components in the amounts and order given in Table I; then shake thoroughly and place in the water bath at 37 C. for one hour. Make readings at the end of this time and note the smallest amount of the guinea-pig serum which completely destroys all the blood cells. This is the "complement unit." For example, in Table I, which shows an actual test, the unit is 0.08 c.c. Keep the complement dilution, when not in use, in the ice box, instead of at room temperature, as it deteriorates easily. In mixing it do not shake excessively.

HEMOLYTIC IMMUNE BODY (AMBOCEPTOR).

The hemolytic immune body (immune rabbit serum) is most commonly derived from rabbits which have been immunized against the blood cells of sheep.

Immunization of Animal. For immunizing purposes, select normal, healthy rabbits weighing from 1800 to 2500 gm. If

*For example, where the immune body serum has a unit value of $\frac{1}{1750}$ c.c. (Table I), $\frac{1}{875}$ c.c. would contain two units; therefore add 0.01 c.c. of this serum to each 8.75 c.c. of 0.9 per cent. salt solution to give the desired value

possible, keep each animal separately caged to prevent fighting and injury. Three animals may be carried through the immunization together. Prepare sheep corpuscles as previously outlined. Pipette off the desired amounts for injection of each animal; place in sterile bottles, and add an equal volume of 0.9 per cent. salt solution to dilute for the injection.

Injection of Animal. Various procedures have been suggested for the immunization of rabbits against sheep blood cells, but experience has shown that none is infallible. Apparently much variation exists between individual animals concerning their ability to form hemolysins. The following procedure has been found, in general, to yield satisfactory results: Fasten a rabbit in the animal holder and expose the marginal ear vein by clipping off the hair and shaving. Pipette 1 c.c. of sterile, packed sheep corpuscles into a small sterile bottle; add an equal volume of sterile 0.9 per cent. salt solution; mix well, and take up in a sterile all-glass syringe through a 3 cm. needle (gauge 21 or 22). Eject any air bubbles present. Grasp the animal's ear firmly with the left hand, and while thus supporting it introduce the needle of the syringe into the vein about 6 to 8 mm. and slowly inject the blood cell suspension. Remove the needle from the vein, and stop the bleeding by compressing that portion of the ear between a fold of sterile absorbent cotton. Repeat this procedure on the second day following, using a dose of 2 c.c. packed corpuscles; then again two days later with a dose of 3 c.c. corpuscles. In each instance use freshly prepared corpuscles and dilute with sterile physiological salt solution.

Sample bleedings may be taken, and the serum tested for hemolytic value, from seven to ten days after the last injection. Usually the antibody content of the blood reaches its maximum concentration at that period. If tests show that the serum is of low titer, the animal may be rejected or more time may be given. If the titer is sufficiently high, collect the blood by placing the animal well under ether and bleeding it to death from the carotid.*

Separation of Serum. Collect about 15 to 18 c.c. of blood aseptically in sterile test-tubes measuring 2.5 x 15 cm.; slant well; and allow to remain at room temperature until firmly clotted. Separate the lower portion of the clot from the side of

* This procedure may be carried out by the method outlined on page 147.

the tube. Place in the ice box over night in an upright position, and the following morning remove the separated serum under aseptic precautions. Place in sterile 15 c.c. centrifuge tubes and throw down free blood cells. Remove the serum and place in well plugged sterile test-tube.

Inactivation of Serum. Place the test-tubes containing the immune serum in water bath at 56 C. for thirty minutes, or longer if the tubes are large. This destroys the complement present, but not the hemolytic immune body (amboceptor).

Standardization of Hemolytic Immune Body. Prepare two dilutions of the inactivated immune serum; the first (1:300) by accurately measuring 0.1 c.c. of the serum by means of a pipette graduated in 1/500 or 1/1000 c.c., adding it to 30 c.c. of 0.9 per cent. salt solution, and thoroughly mixing; the second, (1:3000), by adding 1.0 c.c. of the first dilution to 9.0 c.c. of 0.9 per cent. salt solution. Make a 5 per cent. suspension of sheep blood cells (prepared as for injection purposes) in 0.9 per cent. salt solution. Also prepare fresh standard complement by pooling the serum of at least five guinea-pigs as directed under discussion of complement. Place in a suitable test-tube stand the number of perfectly clean test-tubes (1.2 x 10 cm.) required for the test. Combine the components in the order and amounts indicated in Table II. Shake each tube thoroughly immediately after the blood cell suspension has been added. Place in the water bath at 37 C. for one hour, then remove and note results. Make readings by holding the tube toward the light and observing whether or not a complete destruction of the cells has taken place. If completely hemolyzed, the fluid is clearly transparent, and no residue is left. The smallest amount of the immune body which completely hemolyzes the cells under standard conditions is designated one unit of immune body. Table II shows the results of an actual test on an immune body, which by previous test was found to have a unit value lying somewhere between $\frac{1}{10000}$ and $\frac{1}{20000}$ c.c. As indicated, the unit value was found to be $\frac{1}{17500}$ c.c. Owing to the rather broad limits between the amounts of immune body used in this test, it is well to titrate the same complement back against the standardized immune body, to establish its value in relation to the immune body more accurately in case the same guinea-pig serum is to be used as complement in the Wasser-

mann test at the same time. (For this test, see under Complement).

Preservation and Storage of Immune Body. Sterile immune serum may be preserved for months if placed in sterile ampoules, sealed by flaming the tip, and stored in the dark in a low temperature refrigerator. Usually between 1 and 1.5 c.c. is placed in each ampoule, and the ampoule after sealing is properly labeled, giving titer and date.

ANTIGEN.

It has been found that a number of substances may be used as antigen in the Wassermann test. The original luetic-liver-extract antigen first suggested by Wassermann is seldom used. Perhaps the alcoholic extract of heart (guinea-pig, ox, or human), and the Noguchi acetone-insoluble extract from ox heart are the antigens which are most commonly employed, and in our experience the latter has given very satisfactory results. The selection of any particular antigen depends upon certain requirements, such as a low content of anticomplementary and hemolytic substances, but a high power to bind complement in the presence of serum from luetic patients. Accurate tests must be made on each antigen to measure these factors, and from the results its fitness for practical work is judged.

Preparation of Antigen. The following method, which is a modification of the Noguchi method for the preparation of the acetone-insoluble antigen, has on the whole yielded a satisfactory product. Obtain an ox heart from a freshly slaughtered animal and immediately dissect free from fat, fascia, and tendons. Chop the heart muscle finely by passing twice through a meat chopper. Place the chopped muscle in a meat press, the cylinder of which has been lined with about eight layers of well washed cheese cloth; and apply pressure for a half hour or longer until no more juice can be expressed. Weigh the pressed meat; place it in flasks, and mix with 95 per cent. alcohol in the proportion of 100 gm. to 1000 c.c. Shake the mixture until the lumps are well broken up into small particles. Keep the mixture in a cabinet free from light, at room temperature, for seven to ten days, shaking gently night and morning during that time. Then pour the fluid portion off; collect the residue in washed cheese cloth and express the remaining fluid, filtering the alcoholic solution

through paper. Pour the solution into large shallow dishes to a depth of 1 to 2 cm.; place in a dry room out of the sunlight, and by means of an electric fan evaporate until only a residue remains. Take up the residue in ether so far as soluble. Separate the ether-soluble portion from the insoluble by passing through filter paper. Collect the filtrate in a beaker and allow to evaporate at room temperature until well concentrated. Add the concentrated ether solution to about ten times its volume of chemically pure acetone contained in a tall glass cylinder, while constantly stirring with a glass rod. Allow the resulting precipitate to settle to the bottom of the cylinder; carefully decant the supernatant fluid, and wash the residue two or three times with fresh portions of acetone. Finally wash the residue in a glass evaporating dish, decant the acetone so far as possible, and allow the remainder to evaporate. By means of a spatula, collect the yellow-brown residue, which is the portion used as antigen, into a mass, place in a well fitting, glass-stoppered, wide mouth bottle, and cover with acetone. Stock antigen extract so prepared and stored in the ice box away from the light, will remain unchanged for months.

Testing of Antigen. Besides proving that an antigen possesses strong antigenic properties, it is necessary to prove that it does not contain anticomplementary and hemolytic substances in such amounts as to invalidate its use in practical work. For the purpose of testing an antigen a stock solution of the acetone-insoluble extract is prepared as follows: Place 0.3 gm. of the extract in a strong test-tube and dissolve in 1 to 2 c.c. of ether. To this solution add gradually, while constantly stirring, sufficient chemically pure methyl alcohol to bring the total volume to 10 c.c. The resulting precipitate may be partially dissolved by rubbing out with a glass rod in the solution. Finally remove the residue by filtration. Place the solution in small, dark colored, glass-stoppered bottles and store in the ice box to reserve as stock solution.

Titration of Hemolytic Activity. Make an emulsion of the stock solution, placing in a test tube 1 c.c. of the solution, and to this adding 3 c.c. of 0.9 per cent. salt solution, drop by drop, while constantly shaking. This amount of emulsion is sufficient for both the hemolytic and the anticomplementary titrations which may be carried out at the same time. Arrange in a

suitable stand a series of test tubes like those used in preceding tests, and perform the test as outlined in Table III.

The readings are based on the presence or absence of hemolysis. The maximum amount of the stock solution which shows no hemolysis in the test is taken as its non-hemolytic value, and in the particular instance shown in the table is 0.16 c.c.

Titration of Anticomplementary Power. For this test it is necessary to prepare the following materials: a dilution of hemolytic immune body in 0.9 per cent. salt solution, two units to each c.c.; guinea-pig serum diluted 1:10 in 0.9 per cent. salt solution, then standardized against the immune body dilution to find unit strength; sensitized sheep corpuscles prepared by mixing equal volumes of 5 per cent. corpuscle suspension and the dilution of the immune body (this mixture should stand at room temperature about one hour before using).

Arrange in a stand a series of test-tubes (1.2 x 10 cm.). To these tubes add the emulsion of stock solution, as prepared in the preceding test, in amounts indicated in Table IV (which gives the full procedure to be followed). The maximum amount of the antigen emulsion which shows no inhibition of hemolysis is considered the limit of anticomplementary action. In the table given it is considered as 0.2 c.c.

Titraton of Antigenic Power. If an antigen shows only a small amount of hemolytic and anticomplementary substances, it is tested further to determine its binding power in the presence of luetic sera, and to establish an antigenic unit. This requires at least six or more known positive (++++) luetic sera. In selecting such sera it is necessary to exclude those which in themselves contain anticomplementary substances, also natural hemolytic immune body against sheep corpuscles. This requires preliminary tests upon the positive sera before making tests of the binding power of the antigen. In selecting positive sera for this purpose those showing the presence of bile, fatty substances, hemoglobin, bacterial growth, etc., should be rejected.

To test serum for anticomplementary properties, place a series of test-tubes in a stand, then proceed as outlined in Table V. Dilute the guinea-pig serum 1:10 with salt solution and standardize in the usual way with standard immune body. Also sensitize sheep corpuscles with the same immune body dilution used for standardizing the complement. Those sera (Nos. 3 and 6 in

table) which show inhibition of hemolysis are rejected at once, those passing this test are then tested for the presence of natural immune bodies against sheep corpuscles.

The hemolytic action of the positive luetic sera in the presence of complement is a measure of the amount of natural immune body present. If more than a trace of hemolysis takes place in the tube receiving the larger amount of positive serum, it should be rejected. As in the previous test, standardize guinea-pig serum for complement. Prepare in the regular way a 5 per cent. suspension of sheep corpuscles. Test the positive serum in two amounts as an aid in estimating the amount of natural immune body, if present. Carry out the test as indicated in Table VI. In this case sera No. 5 and No. 6 should be rejected, while serum No. 2 might possibly be used under certain conditions.

For use in the test for the antigenic unit value of the stock solution, pool six or more positive (++++) luetic sera which meet the required conditions. Make emulsions of the stock solution in 0.9 per cent. salt solution by placing 1 c.c. of the stock solution in a test-tube and adding, drop by drop, while constantly shaking, 9 c.c. of the salt solution. From this 1:10 emulsion make another, 1:100, in the same way, and finally from the latter an emulsion of 1:1000. Standardize guinea-pig complement and prepare sensitized sheep corpuscles. Set up a series of test-tubes in a stand as directed in Table VII. Proceed as outlined, using amounts and directions indicated. The least amount of the stock solution (in emulsion) which completely fixes all the complement in the combination is considered one unit. In the case cited the full unit value of the particular antigen falls at tube No. 8, which contains 0.003 c.c. of the original stock solution.

It is well to use if possible ten or more units of antigen in practical work. This type of antigen commonly contains relatively small amounts of hemolytic substances. Therefore, in deciding upon the number of units to be used and in the trial tests, particular attention must be given to the anticomplementary value of the antigen. Never use an amount of the antigen equal to or exceeding half of the anticomplementary amount, as it may cause a false positive reaction. As a general rule, the antigen may be used safely in amounts not exceeding one-third of the anticomplementary dose, provided it contains the required number of units to give complement fixation with positive sera. But

the amount of antigen must not be increased to the extent that marked turbidity of the emulsion obscures the readings of the Wassermann test. Assuming that an antigen whose antigenic unit was found to be 0.003 c.c. was not hemolytic in 0.3 c.c., nor anticomplementary in 0.2 c.c., it could be used safely in twenty units, or 0.06 c.c. If the antigenic value of a stock solution is low, and the anticomplementary value high, these values may approach each other so closely that the antigen is worthless, and must be rejected. All antigens after testing should be run parallel with a good antigen in practical work to prove their value.

Preparation of Patient's Serum, Spinal Fluid, etc. In the preceding paragraphs attention has been given to the preparation and standardization of the biochemical reagents which are used in performing the Wassermann test. It still remains to discuss the preparation of the patient's serum, etc., for test purposes. Collect the blood* in clean, sterile test-tubes; label with patient's name; slant, and allow to remain at room temperature until a firm clot is formed. Loosen the clot from the walls of the tube by means of a strong platinum wire (sterile), and place the tube in the ice box. Each evening remove the sera from the blood samples collected during the day. Remove the serum by means of a pipette; place it in a 15 c.c. conical centrifuge tube; throw down the cells, etc., in the machine. Remove the supernatant fluid; place it in a test-tube; tightly stopper with cork, and then put it in the ice box until time to test. Just previous to testing, inactivate the sera by placing the tubes in the water bath at 55 C. for about thirty minutes.

Draw spinal fluid in the usual way from the patient. If blood cells are present, separate them from the fluid in the centrifuge, and store in ice box until ready for test. Do not inactivate spinal fluid unless evidence of considerable blood is shown, then treat as blood serum. Treat ascitic and other fluids in the same way as spinal fluid.

TECHNIQUE OF REACTION (MODIFIED)

As is evident, the foregoing discussion deals in particular with the materials which must be prepared, and, in several instances, standardized, before the actual Wassermann test can be undertaken. To perform this test the following components must be

* Methods for taking blood are given on page 118.

combined: Patient's serum (spinal fluid, etc.); complement; antigen; sensitized sheep corpuscles, and as diluent, 0.9 per cent. salt solution. Each component must be prepared, standardized, and at hand before starting the test.

In the original Wassermann test the components were combined in such amounts that the final volume was 5 c.c. As a general thing most workers economize materials by doing the test in some aliquot part of the original volume. One-quarter the amount gives satisfactory results, and is used in this laboratory.

It is necessary to have a water bath with a temperature of 37 C. in which the incubation may be carried out. Copper test tube stands with double rows of holes, holding tubes measuring 1.2 x 10 cm. are most suitable for this work.

Make all measurements of materials, in the setting up of the test, with 1 c.c. pipettes divided into $\frac{1}{100}$ c.c. graduations. In measuring the sensitized blood cells use a 5 c.c. pipette graduated in $\frac{1}{10}$ c.c. Set up tubes in the test tube stand by placing two in the front, and one in the back row for each sample of serum or spinal fluid. If serum, add 0.025 c.c. to the first tube in the front row; 0.05 c.c. to the second, and 0.10 c.c. to the back tube. In the case of spinal fluid, add 0.10 c.c. to the first tube, 0.25 c.c. to the second, and 0.5 c.c. to the back tube. Set up controls (positive and negative) in the same way. Since these amounts of serum or spinal fluid are one-quarter the amount used in the full test (5 c.c. volume), use all the other components accordingly. Dilute the guinea-pig serum 1:10 before standardizing, so that the amount of complement which contains one-quarter unit can be used. The amount which just gives complete hemolysis in the complement titration test (one-quarter) is the amount to be used.

Make the antigen emulsion so that 1 c.c. contains the required number of units used for the full test, and use 0.25 c.c. in this case. Prepare the emulsion in the usual way by placing the required amount of the stock solution in a test tube and adding 0.9 per cent. salt solution drop by drop, while constantly shaking, to bring to the required dilution. For example, an antigen has a unit value of 0.003 c.c., and is used twenty units to the c.c. in the full test. In this case each c.c. of the emulsion must contain 0.06 c.c. of the stock solution of antigen. If 30 c.c. of emulsion are required for a given test, then place 1.8 c.c. of the stock solu-

tion in a small flask and add, while constantly shaking, 28.2 c.c. of 0.9 per cent. salt solution. Use 0.25 c.c. to each tube in the one-quarter volume test.

Prepare the sensitized cells as previously mentioned by mixing equal volumes of a hemolytic immune body solution containing two units per c.c. with a 5 per cent. suspension of sheep corpuscles.

In the full test 2 c.c. of this sensitized corpuscle suspension would be used, or 0.5 c.c. in the quarter test. The 0.5 c.c. is equivalent to 0.25 c.c. of 5 per cent. blood suspension and 0.5 unit immune body, which is double the amount some workers use. As a general rule, sheep corpuscles sensitized in this way give sharply defined results. The mixture should be prepared and allowed to stand about one hour before being used in the test.

Add 0.9 per cent. salt solution to bring the total volume in each tube to a constant amount, that is, 0.75 c.c. before the sensitized cells are added, when one-quarter volume test is used.

The order of combining the components varies among different workers. As a routine procedure, pipette the required amount of the serum or spinal fluid into the bottom of the tubes; follow by complement, and salt solution, then shake thoroughly. Next add the antigen, at once shaking thoroughly, and place the tubes in the water bath for incubation. After the first incubation, add the sensitized corpuscles, which must be uniformly mixed. Shake the tubes well and return to the incubator.

Make controls against the antigen by testing it in double the amount used in the test proper to determine if it is anticomplementary in that amount with the system in use. The patient's serum or spinal fluid is omitted in this tube. The hemolytic system is controlled by combining complement, sensitized cells, and diluent to the required volume.

The full procedure with the amounts of components to be used, and the scheme of readings is given in Table VIII.

The reading of the Wassermann reaction is based upon the presence or absence of hemolysis after the test is completed. The degree of hemolysis is inverse in proportion to the degree of complement fixation. The following notation may be used in expressing the different degrees of hemolysis:

C (Complete): Destruction of all blood cells.

AC (Almost complete): Only few blood cells remain.

P (Partial): About one-half blood cells remain.

Sl (Slight): Few blood cells destroyed; supernatant fluid colored slightly red.

O (Negative): No cells destroyed; supernatant fluid water clear.

In marking the strength of the Wassermann reaction, Citron's standard is used as a basis. In order to apply the readings which are used in marking the degree of hemolytic reaction in the test to the Citron standard, the following table has been constructed, and the Wassermann reaction is recorded accordingly:

Hemolysis		Reaction
Tube 1	Tube 2	
O		O = ++++
Incomplete		O = +++
Complete (or almost complete)		O = ++
Complete	Incomplete	= +
Complete	Doubtful	= ±
Complete	Complete	= $\overline{O}$

Key: O: Complete inhibition; no hemolysis.
Sl, P: Incomplete inhibition.
AC: Doubtful inhibition.
C: Complete hemolysis; no inhibition.

In making the readings of the reactions it is well to have the results checked by a second person who is accustomed to the work.

TABLE I.

TITRATION OF COMPLEMENT (ONE-QUARTER UNITS).*

Tube No.	Guinea-pig Serum 1:10 Dilution	Salt Solution 0.9%	Immune Rabbit Serum, 1 c.c.=2 units, e.g., 1:875 Dilution	Sheep Corpuscles 5% Suspension	Water Bath	Hemolysis	Corresponding Amount of Guinea-pig Serum for Whole Units.
	c.c.	c.c.	c.c.	c.c.			c.c.
1	0.3	0.57	0.13	0.25	Incubate 1 hour at 37 C.	Complete	0.12
2	0.25	0.62	0.13	0.25		Complete	0.10
3	0.20	0.67	0.13	0.25		Complete	0.08
4	0.17	0.70	0.13	0.25		Almost Complete	0.068
5	0.13	0.74	0.13	0.25		Almost Complete	0.052
6	0.10	0.77	0.13	0.25		Almost Complete	0.040
7	0.08	0.79	0.13	0.25		Partial	0.032
8	0.06	0.81	0.13	0.25		Slight	0.024
9	—	0.87	0.13	0.25		None	Control

*Modified from Wood, Chemical and Microscopical Diagnosis, 3d ed., 1912.

TABLE II.

TITRATION OF HEMOLYTIC SERUM (ONE-QUARTER UNITS).*

Tube No.	Immune Rabbit Serum	Salt Solution 0.9%	Guinea-pig Serum, 1:10 Dilution	Sheep Corpuscles 5% Suspension	Water Bath	Hemolysis	Corresponding Dilution for Whole Units
	c.c. 1-300	c.c.	c.c.	c.c.			
1	0.75	0.25	0.00	0.25	Incubate 1 hour at 37 C.		Control
2	0.15	0.60	0.25	0.25			1/500
3	0.10	0.65	0.25	0.25			1/750
	1-3000						
4	0.75	0.00	0.25	0.25		Complete	1/1000
5	0.60	0.15	0.25	0.25		Complete	1/1250
6	0.50	0.25	0.25	0.25		Complete	1/1500
7	0.43	0.32	0.25	0.25		Complete	1/1750**
8	0.38	0.37	0.25	0.25		Almost Complete	1/2000**
9	0.33	0.42	0.25	0.25			1/2250**
10	0.30	0.45	0.25	0.25			1/2500
11	0.27	0.48	0.25	0.25			1/2750**
12	0.25	0.50	0.25	0.25			1/3000
13	0.21	0.54	0.25	0.25			1/3500**
14	0.00	0.75	0.25	0.25			Control

*Modified from Wood.

**Approximately correct.

TABLE III.

TITRATION OF HEMOLYTIC ACTIVITY OF ANTIGEN STOCK SOLUTION.* (ONE-QUARTER UNITS).

Tube No.	Antigen Stock Solution	Salt Solution 0.9%	Sheep Corpuscles 5% Suspension	Water Bath	Hemolysis	Amount of Antigen Stock Solution for Whole Unit
	c.c. Emulsion 1-4	c.c.	c.c.			c.c.
1	0.4	0.6	0.25	Incubate 1 hour at 37 C.	Partial	0.4
2	0.3	0.7	0.25		Partial	0.3
3	0.2	0.8	0.25		Slight	0.2
4	0.16	0.84	0.25		None	0.16
5	0.1	0.9	0.25		None	0.1
6	0.06	0.94	0.25		None	0.06
7	0.04	0.96	0.25		None	0.04
8	0.00	1.00	0.25		None	Control

*Modified from Wood.

TABLE IV.

TITRATION OF ANTICOMPLEMENTARY POWER OF ANTIGEN STOCK SOLUTION (ONE-QUARTER UNITS).*

Tube No.	Antigen Stock Solution	Salt Solution 0.9%	Guinea-pig Serum 1-10 Dilution	Water Bath	Sensitized Corpuscles 1 c.c. = 1 unit	Water Bath	Hemolysis	Amount of Antigen Stock Solution for Whole Unit.
	c.c. Emulsion 1-4	c.c.	c.c.		c.c.			c.c.
1	0.4	0.1	0.25	Incubate 1 hour at 37 C.	0.5	Incubate 1 hour at 37 C.	None	0.4
2	0.3	0.2	0.25		0.5		Marked	0.3
3	0.2	0.3	0.25		0.5		Complete	0.2
4	0.16	0.34	0.25		0.5		Complete	0.16
5	0.1	0.4	0.25		0.5		Complete	0.1
6	0.06	0.44	0.25		0.5		Complete	0.06
7	0.04	0.46	0.25		0.5		Complete	0.04
8	0.00	0.5	0.25		0.5		Complete	Control

*Modified from Wood.

TABLE V.

TITRATION OF POSITIVE LUETIC SERA FOR ANTICOMPLEMENTARY SUBSTANCES (ONE-QUARTER UNIT).

Specimen	Positive Serum	Guinea-pig Serum 1:10 Dilution	Salt Solution 0.9%	Water Bath	Sensitized Corpuscles 1 c.c.=1 unit	Water Bath	Hemolysis*
	c.c.	c.c.	c.c.		c.c.		
1	0.10	0.2*	0.45		0.5		Complete
2	0.10	0.2	0.45		0.5		Complete
3	0.10	0.2	0.45		0.5		Almost Complete
4	0.10	0.2	0.45		0.5		Complete
5	0.10	0.2	0.45		0.5		Complete
6	0.10	0.2	0.45		0.5		Slight
7	0.10	0.2	0.45		0.5		Complete
8	0.10	0.2	0.45		0.5		Complete
9	0.10	0.2	0.45		0.5		Complete
10	0.10	0.2	0.45		0.5		Complete
11	0.10	0.2	0.45		0.5		Complete
12	0.10	0.2	0.45		0.5		Complete
Control	0.0	0.2	0.55		0.5		Complete

Incubate 1 hour at 37 C.

Incubate 30 minutes at 37 C.

*Variable, depending upon standardization.

TABLE VI

TITRATION OF POSITIVE LUETIC SERA FOR NATURAL IMMUNE BODY AGAINST SHEEP CORPUSCLES (ONE-QUARTER UNITS).

Specimen	Positive Serum	Guinea-pig Serum 1:10 Dilution	Sheep Corpuscles 5% Suspension	Salt Solution 0.9%	Water Bath	Hemolysis
	c.c.	c.c.	c.c.	c.c.		
1	0.025	0.2*	0.25	0.775		O
	0.05	0.2	0.25	0.75		O
2	0.025	0.2	0.25	0.775		O
	0.05	0.2	0.25	0.75		Very Slight
3	0.025	0.2	0.25	0.775		O
	0.05	0.2	0.25	0.75		O
4	0.025	0.2	0.25	0.775		O
	0.05	0.2	0.25	0.75		O
5	0.025	0.2	0.25	0.775	Incubate 1 hour at 37 C.	Slight
	0.05	0.2	0.25	0.75		Almost Complete
6	0.025	0.2	0.25	0.775		Almost Complete
	0.05	0.2	0.25	0.75		Complete
7	0.025	0.2	0.25	0.775		O
	0.05	0.2	0.25	0.75		O
8	0.025	0.2	0.25	0.775		O
	0.05	0.2	0.25	0.75		O
9	0.025	0.2	0.25	0.775		O
	0.05	0.2	0.25	0.75		O
10	0.025	0.2	0.25	0.775		O
	0.05	0.2	0.25	0.75		O
Compl. Control No. 1	—	0.2	0.25	0.8		O
Compl. Control No. 2	—	0.4	0.25	0.6		O

*Variable, depending upon standardization.

TABLE VII.

TITRATION OF ANTIGEN STOCK SOLUTION FOR UNIT VALUE. (ONE-QUARTER UNITS).

Tube No.	Positive Serum (pooled)	Guinea-pig Serum 1:10 Dilution	Antigen Dilutions	Salt Solution 0.9%	Water Bath	Sensitized Corpuscles	Water Bath	Hemolysis	Whole Antigen Unit for Stock Solution
	c.c.	c.c.	c.c.	c.c.		c.c.			c.c.
			1:10 emul.						
1	0.05	0.2*	0.5	0		0.5		None	0.2
2	0.05	0.2	0.25	0.25		0.5		None	0.1
3	0.05	0.2	0.15	0.35		0.5		None	0.06
4	0.05	0.2	0.075	0.425		0.5		None	0.03
5	0.05	0.2	0.05	0.45		0.5		None	0.02
			1:100 emul.						
6	0.05	0.2	0.25	0.25		0.5		None	0.01
7	0.05	0.2	0.15	0.35		0.5		None	0.006
8	0.05	0.2	0.075	0.425		0.5		None	0.003
9	0.05	0.2	0.05	0.45		0.5		Slight	0.002
			1:1000 emul.						
10	0.05	0.2	0.25	0.25		0.5		Partial	0.001
11	0.05	0.2	0.15	0.35		0.5		Almost Complete	0.0006
12	0.05	0.2	0.075	0.425		0.5		Complete	0.0003
13	0.05	0.2	0.05	0.45		0.5		Complete	0.0002
14									
Serum Control 15	0.10	0.2	—	0.45		0.5		Complete	
			1:10 emul.						
Antigen Control 16	—	0.2	0.5	0.05		0.5		Complete	
Hemolytic System	—	0.2	—	0.55		0.5		Complete	

Incubate 1 hour at 37 C.

Incubate 1 hour at 37 C.

*Variable, depending upon standardization.

TABLE VIII. COMPOSITE TABLE SHOWING WASSERMANN TEST WITH READINGS. (ONE-QUARTER UNITS.)

		FRONT ROW									BACK ROW						
Tube No.	Test Substance	Amounts of Test Fluid	Guinea-pig Serum 1-10 Dilution	Salt Solution 0.9%	Antigen Dilution**	Water Bath	Sentitized Corpuscles 1 c.c.=1 unit	Water Bath	Hemolysis	Reaction	Amount of Test Fluid	Guinea-pig Serum 1-10 Dilution	Salt Solution 0.9%	Water Bath	Sensitized Corpuscles 1 c.c.=1 unit	Water Bath	Hemolysis
		c.c.	c.c.	c.c.	c.c.		c.c.				c.c.	c.c.	c.c.		c.c.		
1	Serum, 1	0.025	0.2*	0.275	0.25		0.5		None	Positive	0.10	0.2	0.45		0.5		Complete
2		0.05	0.2	0.25	0.25		0.5		None	(++++)							
3	" 2	0.025	0.2	0.275	0.25		0.5		Partial	Positive	0.10	0.2	0.45		0.5		Complete
4		0.05	0.2	0.25	0.25		0.5		None	(+++)							
5	" 3	0.025	0.2	0.275	0.25		0.5		Almost Complete	Positive	0.10	0.2	0.45		0.5		Complete
6		0.05	0.2	0.25	0.25		0.5		None	(++)							
7	" 4	0.025	0.2	0.275	0.25		0.5		Complete	Positive	0.10	0.2	0.45		0.5		Complete
8		0.05	0.2	0.25	0.25		0.5		Slight	(+)							
9	" 5	0.025	0.2	0.275	0.25		0.5		Complete	Doubtful	0.10	0.2	0.45		0.5		Complete
10		0.05	0.2	0.25	0.25		0.5		Almost Complete	(+)							
11	" 6	0.025	0.2	0.275	0.25		0.5		Complete	Negative	0.10	0.2	0.45		0.5		Complete
12		0.05	0.2	0.25	0.25		0.5		Complete	(o)							
13	" 7	0.025	0.2	0.275	0.25		0.5		Almost Complete	Indefinite	0.10	0.2	0.45		0.5		Almost Complete
14		0.05	0.2	0.25	0.25		0.5		Slight	(Anticomp.)							
15	Spinal fluid, 8	0.10	0.2	0.20	0.25		0.5		Complete	Positive	0.5	0.2	0.05		0.5		Complete
16		0.25	0.2	0.05	0.25		0.5		None	(++)							
17	" " 9	0.10	0.2	0.10	0.25		0.5		Almost Complete	Positive	0.5	0.2	0.05		0.5		Complete
18		0.25	0.2	0.05	0.25		0.5		Partial	(+)							
19	Positive Serum	0.025	0.2	0.275	0.25		0.5		None	Positive	0.10	0.2	0.45		0.5		Complete
20	Control	0.05	0.2	0.25	0.25		0.5		None	(++++)							
21	Negative Serum	0.025	0.2	0.275	0.25		0.5		Complete	Negative	0.10	0.2	0.45		0.5		Complete
22	Control	0.05	0.2	0.25	0.25		0.5		Complete	(o)							
23	Antigen Control	0	0.2	0.05	0.50		0.5		Complete								
24	Hemolytic System	0	0.2	0.55	0		0.5		Complete								

Incubate 1 hour at 37 C. (Front Row)

Incubate 30 minutes at 37 C. (Front Row)

Incubate 1 hour at 37 C. (Back Row)

Incubate 30 minutes at 37 C. (Back Row)

*Variable, depending upon standardization.

**Each c.c. of dilution may contain 10 or more units, but in amount safely below the anticomplementary or hemolytic dose for the system in use.

WIDAL TEST.

Collection of Blood. Usually blood for the Widal test is collected from the finger or ear, although when a culture also is ordered, blood for the two may be drawn at the same time. For the small amount of serum required, prick the finger or ear; touch the curved end of a Wright capsule to the drop; and hold the capsule so that the shaft slants downward at an angle of about 45 degrees. Collect to a depth of about 2 cm. in tube of 5 mm. diameter. Seal in flame, first the curved, then the straight tip of the tube. The capsule may be placed in a carrier, and the clear serum separated from clot in the centrifuge.

Culture of Organism. Each day prepare a fresh culture of *B. typhosus,* either by inoculating a broth tube from fresh agar slant growth and incubating, or by adding physiological salt solution to the agar slant growth and making a thin suspension of the organisms. It is essential that the culture be motile and have no clumps. Do not use cultures which are old; young cultures give best results.

Apparatus Required. Clean cover-slips; hollow ground slides; capillary (Pasteur) pipettes; and a clean porcelain color-mixing tray (such as used in mixing water colors).

Procedure. File and break Wright capsule and set up so that serum may be removed with capillary pipette. Place color-mixing tray in a larger enamel-ware tray, and fill gutter of color tray with physiological salt solution. With vaseline rim margin about well in hollow ground slides. Place clean cover-slips on small strip of blotting paper. Place small rubber nipple on capillary pipette, which should be plugged with cotton. Take up serum in capillary portion of the pipette to a distance of 5 cm. or more, and put one drop in first cup of tray. Empty the pipette and rinse with salt solution. Add nine drops of salt solution to the drop of serum, and mix by drawing in and out of the empty pipette. Put five drops of this mixture into second cup, emptying surplus from the pipette back into the first cup; then rinse pipette in salt solution; and add five drops of salt solution to second cup. Again take five drops from second cup and mix with five drops of salt solution in third. This gives dilutions

1:10, 1:20, and 1:40. Take a portion of the broth culture in the same pipette, and add five drops to the first two cups, and ten to the third, making the dilutions 1:20, 1:40 and 1:80. Make a hanging drop from each dilution by taking a small drop with cool sterile platinum loop and depositing on cover-slip which is placed on a small blotter. Invert slide over coverslip so that the drop comes in center of well, then gently press slide down on cover until the two are firmly sealed by the vaseline ring. Raise, and turn slide over quickly so that the cover-slip is uppermost. Label each slide with patient's name, the dilution, and the time. Make a control of the culture by mixing one loopful with one loopful of the physiological salt on a cover-slip; mount in the same way. This method requires four cover-slips, and the same number of slides. By mounting all the drops on one cover-slip, time as well as slides may be saved, although great care must be exercised not to allow drops to run together, and to avoid confusion in labeling. To proceed, select hollow ground slide with a well of large diameter, ring wtih vaseline. With blue pencil label upper left side from well "control", lower left side "1:40"; upper right side "1:20", lower right "1:80", and add patient's name. Place a cover-slip on small blotter, label blotter with pencil, respective to position of cover-slip, upper left side "1:40", lower left "control", upper right side "1:80", lower right "1:20". With small platinum loop place drops from dilutions and the control in their respective positions, each as near the center as possible without running together. Invert hollow ground slide over the cover-slip so that the corresponding labels fall in the correct positions; seal together by gentle pressure; lift up and turn slide so that the cover-slip is uppermost. After making the hanging drops, keep for one hour at room temperature out of the direct sunlight; then examine with No. 6 or No. 7 lens. Note the amount of clumping and motility. For the sake of uniformity use the following terms:

CLUMPING	*MOTILITY*
None	Unimpaired
Slight	Active
Fair	Fair
Good	Slightly persistent
Almost complete	None
Complete	

The test is worthless if the control shows agglutination at the end of an hour; but a test on non-motile but unagglutinated bacteria gives results of considerable value.

Enter the results in the bacteriological record book, and on the daily record sheet, according to the form given in the following example:

Widal Control: no clumping; activity motile
1:20; good clumping; Slightly persistent motility.
1:40; good clumping; slightly persistent motility.
1:80; good clumping; fair motility.

Test on Dried Blood. To one drop of blood dried on a slide add one drop of physiological salt solution. Rub up with a platinum loop until blood is dissolved as well as possible. Transfer to cup of tray, and add four drops of the salt solution; mix; transfer three drops to second cup; and add three drops of salt solution. Mix; transfer three drops to third cup: and add three drops of salt solution. Add broth culture, two drops to the first cup; three drops to the second cup, six to the third cup. Make hanging drops from each, also make a control; mount as outlined above; and examine.

Always take precautions to prevent spreading of the live typhoid culture; work over large enamel-ware tray containing color tray, etc. Place capillary pipette, used in handling culture, in cylinder on table containing 2.5 per cent. cresol solution; with the small rubber bulb draw into pipette, before submerging it, some of the disinfecting solution. Place the Wright capsule, after finishing, in the boiler for sterilization. Cover color tray as soon as hanging drops are taken for test. When test is finished remove cover and flood the color tray and the bottom of the large work tray containing it with 2.5 per cent. cresol solution, and allow them to stand some hours to disinfect before cleaning. After microscopical readings are made, place the slide and cover intact in boiler for sterilization, or boil in sterilizer at once.

DILUTION OF DIPHTHERIA TOXIN FOR SCHICK REACTION.

The standardized concentrated diphtheria toxin labelled with M. L. D. (minimum lethal dose for guinea-pigs) is procured from the Research Laboratory, Department of Health, New York. It is kept

in ice-box protected from light, and under such conditions retains its strength for a considerable time.

Make a stock dilution from the concentrated toxin by adding sufficient amount of the latter to 0.25 per cent. tricresol solution so that 1 c.c. contains 10 M. L. D. Of this stock solution prepare 10 c.c. each time; place in a sterile amber glass bottle; seal with a sterile rubber stopper; and store in ice-box free from light. Reject the stock solution after two weeks, since owing to deterioration it loses its value.

For the actual test prepare a second dilution by adding 0.1 c.c. of the first or stock solution to 9.9 c.c. sterile physiological salt solution. Place this dilution in a sterile amber glass bottle, seal with sterile rubber stopper, and label "Diphtheria toxin dilution for Schick reaction, 0.2 c.c., contains 1/50 M. L. D.," and add the date of preparation.

The final dilution for the test should not be used later than twenty-four hours after its preparation, and should be kept in the ice-box in dark except while in use. Strict attention should be paid to the original M. L. D. value of each sample of concentrated diphtheria toxin supplied, as this is not constant. Also note carefully the directions which are usually furnished for the preparation of the dilutions.

DILUTION OF LUETIN.

Luetin may be procured already prepared in proper dilution for administration. In such case, when ordered for the ward, after shaking mixture thoroughly, with sterile pipette remove 0.5 to 1 c.c. from the original package, which is kept in the ice-box, and place in a 5 c.c. sterile bottle; seal the bottle with a sterile rubber stopper, then label properly, stating amount to be administered, and the date of preparation. Sometimes luetin (Rockefeller Institute) is supplied as a concentrated emulsion, and must be diluted with sterile physiological salt solution before using. Make the strength of the dilution according to directions furnished. Prepare only small amounts at a time, place in bottle, label, etc., as mentioned above. Always shake bottle thoroughly before removing emulsion to make the dilution. Since the amount to be administered is variable, the directions received with the orginal package must be carefully noted and followed. Keep the dilution in the ice-box when not in use.

B. tuberculosis
B. smegmatis
B. lepræ
B. Mölleri (grass bacillus)
B. butyricus (butter bacillus of Rabinovitsch)
Some bacteria occurring in distilled water
Some types of Streptothrices

GROUPS OF BACTERIA.

Classified according to Gram Stain.

Positive

Cocci:

M. tetragenus
Pneumococcus group
Staphylococcus group
Streptococcus group

Bacilli:

B. aërogenes capsulatus
B. anthracis
B. botulinus
B. diphtheriæ group
B. tetani
B. tuberculosis and other acid-fast bacilli

Negative

Cocci:

M. catarrhalis
M. gonorrhœæ
M. intracellularis meningitidis and related types
M. melitensis
M. pharyngis siccus
Micrococci of chromogenic group

Bacilli:

B. acidi lactici
B. cloacæ
B. coli group
B. dysenteriæ group
B. enteritidis
B. fæcalis alkaligenes
B. influenzæ group
B. Koch-Weeks
B. lactis aërogenes
B. maligni œdematis
B. mallei
B. Morax-Axenfeld
B. mucosus capsulatus
B. pertussis group
B. pestis
B. proteus
B. pyocyaneus
B. typhosus group

Spirillum:

S. choleræ

FERMENTATION REACTIONS OF GRAM-NEGATIVE GROUP OF COCCI ON SUGAR SERUM-WATERS*

Strains	Dextrose	Maltose	Levulose	Saccharose	Lactose	Galactose
Meningococcus	+	+	O	O	O	O
Pseudomeningococcus	+	+	O	O	O	O
Gonococcus	+	O	O	O	O	O
Micrococcus catarrhalis	O	O	O	O	O	O
Micrococcus pharyngis siccus	+	+	+	+	O	O
Chromogenic group I	+	+	+	+	O	O
Chromogenic group II	+	+	+	O	O	O
Chromogenic group III	+	+	O	O	O	O

*Abridged from Elser and Huntoon.
+ = fermentation
O = no fermentation.

TABLE FOR DILUTION OF "OLD TUBERCULIN" OF KOCH
(After Park and Williams)

Dilutions	Amount of Tuberculin	Amt. of diluent*	Content of tuberculin terms of finished product
A	1 c.c.	9 c.c.	1 c.c. = 0.1 c.c. or gm., or 100 c.mm. or mgm.
B	1 c.c. dilution A	9 c.c.	1 c.c. = 0.01 c.c. or gm., or 10 c.mm. or mgm.
C	1 c.c. dilution B	9 c.c.	1 c.c. = 0.001 c.c. or gm., or 1 c.mm. or mgm.
D	1 c.c. dilution C	9 c.c.	1 c.c. = 0.0001 c.c. or gm., or 0.1 c.mm. or mgm.
E	1 c.c. dilution D	9 c.c.	1 c.c. = 0.00001 c.c. or gm., or 0.01 c.mm. or mgm.
F	1 c.c. dilution E	9 c.c.	1 c.c. = 0.000001 c.c. or gm., or 0.001 c.mm. or mgm.

*The dilutions should be made with sterile physiological salt solution containing about 0.25 per cent. tricresol.

FERMENTATION REACTIONS OF B. DIPHTHERIÆ GROUP*

HISS MEDIUM (TEN DAYS' GROWTH)

ORGANISM	Glucose	Lactose	Saccharose	Galactose	Maltose	Levulose	Mannit.	Dextrin	Glycerin
B. diphtheriæ, virulent and avirulent	C A	C A	O	C A	C A	C A	O	C A	C A
Hoffmann bacillus..	O	O	O	O	O	O	O	O	O
Xerosis bacillus...	C A	O	O	O	O	C A	O	O	C A
B. coryzæ.....	C A	O	O	C A	O	C A	O	O	O

C = coagulation
A = acid
O = no reaction

*Abridged from Graham-Smith.

FERMENTATION REACTIONS‡ OF A GENERAL GROUP OF GRAM-NEGATIVE BACILLI

Organism	Dextrin	Dextrose	Galactose	Lactose	Levulose	Maltose	Mannit	Saccharose	Litmus Milk	Gelatine Liquefaction	Indol	Motility
B. acidi lactici	++	++	++	++	++	++	++	—	+ c*	—	+*	—
B. cloacæ	++	++	++	++	++	++	++	++	c p	+	—	+
B. coli communis	++	++	++	++	++	++	++	—	c	—	+	+
B. coli communior	++	++	++	++	++	++	++	++	c	—	+	+
B. dysentery (Shiga)	—	+	—	—	—	—	—	—	+ —	—	—	—
B. dysentery (Flexner)	+	+	+	—	+	+	+	+	+ —	—	+	—
B. dysentery (Hiss-Russell)	—	+	+	—	+	—	+	—	+ —	—	+	—
B. dysentery (Rosen)	+	+	+	+	+	+	+	—	+	—	+	+
B. enteritidis	++	++	++	—	++	++	++	—	—	—	—	+
B. fæcalis alkaligenes	—	—	—	—	—	—	—	—	—	—	—	+
B. lactis aérogenes	++	++	++	++	++	++	++	++	c	—	+	—
B. mucosus capsulatus	++	++	++	++	++	++	++	++	c	—	+	—
B. Morgan No. 1	?	++	?	—	?	?	—	—	+ —	—	?	+
B. paracolon (Day)	++	++	++	—	++	++	++	—	+ —	—	?	+
B. paratyphosus, alpha	—	++	++	—	++	++	++	—	+	—	—	+
B. paratyphosus, beta	—	++	++	—	++	++	++	—	+ —	—	—	+
B. proteus	?	++	++	—	++	++	—	++	p	+	+	+
B. pyogenes fœtidus	?	+	+	+	+	+	+	+	+	—	?	+
B. typhosus	+	+	+	—	+	+	+	—	+ —	—	—	+

‡Exceptions in some details may be encountered among certain strains of these organisms.

*Usually

Carbohydrate media

— = no fermentation
+ = acid formation
++ = acid and gas formation

Nomenclature

Milk

— = no fermentation, alkaline reaction
+ = acidity with terminal alkalinity
+ = acid
c = coagulation
p = peptonization